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Lethal and sublethal synergistic effects of a new systemic pesticide, flupyradifurone (Sivanto®), on honeybees

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The honeybee (*Apis mellifera* L.) is an important pollinator and a model for pesticide effects on insect pollinators. The effects of agricultural pesticides on honeybee health have therefore raised concern. Bees can be exposed to multiple pesticides that may interact synergistically, amplifying their side effects. Attention has focused on neonicotinoid pesticides, but flupyradifurone (FPF) is a novel butenolide insecticide that is also systemic and a nicotinic acetylcholine receptor (nAChR) agonist. We therefore tested the lethal and sublethal toxic effects of FPF over different seasons and worker types, and the interaction of FPF with a common SBI fungicide, propiconazole. We provide the first demonstration of adverse synergistic effects on bee survival and behaviour (poor coordination, hyperactivity, apathy) even at FPF field-realistic doses (worst-case scenarios). Pesticide effects were significantly influenced by worker type and season. Foragers were consistently more susceptible to the pesticides (4-fold greater effect) than in-hive bees, and both worker types were more strongly affected by FPF in summer as compared with spring. Because risk assessment (RA) requires relatively limited tests that only marginally address bee behaviour and do not consider the influence of bee age and season, our results raise concerns about the safety of approved pesticides, including FPF. We suggest that pesticide RA also test for common chemical mixture synergies on behaviour and survival.

1. Introduction

Pollinators provide ecosystem services that are crucial for crop production and wild plant biodiversity [1]. The honeybee is a major pollinator [2] whose global decline in health raises concerns about ecological impacts, including food security and human welfare [3]. Multiple studies have focused on honeybees because their general biochemistry and neurophysiology are better known than other pollinators and since bees can be used to model pesticide harm to other insect pollinators [4]. Pesticides are among the most important stressors affecting bee health [5] and pose heightened risks when bees are exposed to multiple pesticides for extended periods [6]. Attention has focused on the neonicotinoid pesticides [7], but their use has been progressively restricted because of their adverse effects on bees [8], and growing pesticide resistance [9]. New pesticides, such as flupyradifurone (FPF, Sivanto®, Bayer CropScience AG [10]), have therefore entered the market [9].

FPF is a newly developed systemic insecticide [11] that shares multiple similarities with the neonicotinoids. Its chemical structure partially overlaps with neonicotinoids, but FPF is a butenolide insecticide because of its different pharmacophore [12]. Nonetheless, they share the same target site (agonists of

insects nAChRs, Insecticide Resistance Action Committee (IRAC) group 4), are both systemic [12] and control a wide variety of pests [12] on diverse crops through multiple application methods [10,12]. FPF metabolites include 6-chloronicotinic acid, which is also a metabolic by-product of most neonicotinoids [9], and can cause adverse oxidative stress in organisms such as freshwater amphipods and algae [13].

Because FPF is relatively new, fewer pest species are resistant to it as compared with the neonicotinoids [11,12,14]. FPF is also thought to have a favourable ecotoxicological safety profile [12] and is defined as relatively 'bee safe' [15]. Consequently, while neonicotinoids can only be used to treat crops in the absence of bee foraging, FPF can be used on flowering crops when bees are actively foraging.

Relatively few studies have investigated the impacts of FPF on bees. Hesselbach & Scheiner [16] showed that acute exposure to a high, non-field-realistic FPF dose (1.2 µg bee^{-1}) impaired bee taste and cognition. Tan *et al.* [17] demonstrated that chronic exposure to FPF impaired olfactory learning in larval (0.033 µg larvae d^{-1}) and adult (0.066 µg adult bee d^{-1}) Asian honeybees (*Apis cerana*) at field-realistic doses. Campbell *et al.* [18] tested the effects of FPF in a USA field study and observed no significant side effects on bee colony strength. This latter study, however, shows the difficulty of performing ecotoxicological field trials [19]: bee-collected nectar and pollen sampled from control fields were also contaminated with FPF.

Synergistic effects occur when combined exposure to two factors results in an effect that is significantly greater than the sum of individual effects. Such synergistic effects can occur between pesticide and poor nutrition, reducing bee survival, food consumption and energy levels [20]. Synergy can also arise between a pesticide and disease [21] or from exposure to multiple pesticides [22]. Chemical mixtures can have sublethal effects that do not immediately reduce survival [23,24]. For example, pesticide mixtures can synergistically alter behaviour such as mobility in aquatic organisms [25,26]. The combination of diseases (fungi) and pesticide (imidacloprid) can synergistically alter beetle movement [27]. However, there is, to date, no evidence that pesticides have significant synergistic effects on pollinator behaviour.

Neonicotinoids and SBI (sterol biosynthesis inhibitor) fungicides have synergistic effects on bees because SBI fungicides can inhibit detoxification [28]. FPF and the fungicide, tebuconazole, decreased FPF LD_{50} of in-hive bees by 6-fold [15]. The SBI fungicide propiconazole (PRO; chemical group: triazole; MoA code: G1, DeMethylation Inhibitors (DMI), SBI class 1; Fungicide Resistance Action Committee (FRAC) code: 3) is one of the most commonly used fungicides and is found in the environment and in bee food [29–32]. Both FPF and PRO are used on the same common crops [33]. As FPF is a relatively new pesticide, no monitoring studies have yet tested its co-occurrence as an environmental contaminant with other pesticides. However, FPF is approved for many of the same crops as the neonicotinoids [33], and neonicotinoids co-occur with SBI fungicides in bee pollen [6]. We therefore investigated the potential synergistic effects of two systemic pesticides, FPF and PRO.

We also examined the effects of seasonality. Although summer bees are typically more sensitive to pesticides than winter bees [34–37], some studies have reported different results [35,38]. Baines *et al.* [38] showed that early spring bees (March) were more susceptible to pesticides than summer bees. Decourtye *et al.* [35] found that exposure to a neonicotinoid pesticide (imidacloprid) reduces survival of winter bees, but reduced learning performances of summer bees. Seasons influence the floral and food resources available, thereby altering pesticide resistance [20], toxicokinetics and bee immunity [39]. Pesticide effects are also temperature dependent and alter thermoregulation [40], and season could alter bee detoxification abilities [39].

The effects of pesticides can be influenced by the age and body weight of an organism [39,41,42]. In-hive bees are typically heavier and more resistant to pesticides than foragers [43]. Bee responses to toxins also change as they age [44–48], and older bees may be more sensitive to pesticides [34]. However, to date, no studies have examined how the toxicity of FPF varies across worker type and season. We thus tested the effects of FPF over seasons and between worker types, assessing its interactive effects with a common SBI fungicide, PRO, on bee behaviour and survival.

2. Material and methods

We used six healthy honeybee colonies (*Apis mellifera ligustica* Spinola, 1806, located at the UCSD Biology Field Station apiary, La Jolla, USA), studied forager and in-hive honeybees, and followed standard collection and rearing methodologies [49]. To test the effect of season, we collected bees at two different colony developmental stages: early spring (February–March 2016) and summer (July 2016). We tested the synergistic and individual effects of FPF exposing bees to five acute oral doses of FPF or FPF + PRO. Based on current guidelines [50,51], we tested FPF doses (375 and 750 ng bee^{-1}) considered field-realistic, since bees can ingest higher FPF doses while foraging (see electronic supplementary material for the worst-case scenario estimations).

Following previous studies [22,28,52], we used a relatively high PRO dose that nonetheless, on its own, has no impact on bee survival (7000 ng bee^{-1} [22,52]). PRO is one of the most commonly used fungicides that contaminates bees and the environment [31,32]. Bees can be simultaneously exposed to FPF and PRO (or another SBI fungicide with similar mode of action) because they are used on the same crops and ornamentals, including fruits (e.g. citrus), oilseeds (e.g. soya bean, peanuts), cereals (e.g. corn, sorghum) [10,12,15,53–55], although guidelines state that flupyradifurone should not be directly tank-mixed with azole fungicides when applied to flowering crops [10]. These pesticides can be used multiple times over a year in the same crop (and over different seasons) and applied in multiple ways (i.e. aerial, chemigation or ground application). In addition, bees can also be exposed to pesticides that drift from different crops (i.e. buffer zones) or are stored in the same hive [56,57]. FPF and PRO are easily taken up by plants and thus contaminated soil and water may lead to unintended absorption. This can result in prolonged, multi-year contamination [56,58,59]. Bees can therefore be exposed to pesticide combinations that are contraindicated in tank mixes [6].

We tested a control dose (0 ng bee^{-1}), a total of six doses of FPF (375, 750, 1500, 3000, 6000, 12 000 ng bee^{-1}, respectively corresponding to 37.5, 75, 150, 300, 600, 1200 ppm), and five doses of the positive control dimethoate (DIM; 50, 100, 200, 400, 800 ng bee^{-1}, respectively corresponding to 5, 10, 20, 40, 80 ppm). In the combined FPF + PRO treatment, each FPF dose was tested in combination with a single sublethal dose of PRO (7000 ng bee^{-1}, corresponding to 700 ppm). We used technical grades of all active ingredients. The test solutions (sucrose 50% w/w, 100 µl cage^{-1}, 10 µl bee^{-1},) were provided inside each cage using an Eppendorf cap [60], contained acetone as a solvent (0.7%) and were completely consumed 60 min after oral administration [60].

We measured the effects of treatment on bee survival (1–48 h) and the frequency of bees exhibiting abnormal behaviours (1–4 h, see below).

Detailed methods are reported in the electronic supplementary material.

(a) Abnormal behaviours: synergistic and individual effects

We measured the percentage of bees exhibiting abnormal behaviours (i.e. the number of abnormally behaving bees per cage) across time (1, 2 and 4 h after treatment) depending on pesticide dose, season and worker type. We quantified the following behaviours: motion coordination deficits, hyperactivity, apathy, curved-down abdomen or moribund (electronic supplementary material, table S1) [4,15,51,60–62]. These abnormal behaviour categories are based on official ecotoxicological guidelines [60,63]. The unit of replication was the cage, and we observed each bee for 6 s (a maximum of 60 s for a cage with 10 bees). To improve the standardization and repeatability of our behavioural assessments, we refined the accuracy of the definitions of the behaviours of the official ecotoxicological guidelines [60,63] through videos (electronic supplementary material) and descriptions (electronic supplementary material, tables S1 and S2). We measured abnormal behaviours up to 4 h because high mortality at later time points severely reduced sample sizes in certain treatments and because behavioural abnormalities primarily occurred less than 4 h after treatment. The experimenters were blind to the treatments and were trained using standard descriptions (electronic supplementary material, tables S1 and S2) and videos (electronic supplementary material) of the behaviours. Before being allowed to score behaviours, experimenters needed to score standard videos with greater than 95% consistency.

(b) Statistical analysis

To test for synergy, we determined if the difference between the expected and the observed effects (either mortality or presence of abnormal behaviour) of the combined treatment could arise by chance alone or was larger than the simple additive effect of both pesticides.

We used the concentration addition (CA) reference model to define biologically significant synergy of chemical mixtures [64]. Based on each worker type LD_{50}, we calculated the model deviation ratio (MDR) to determine if the FPF + PRO interaction caused synergistic (MDR > 2), additive ($0.5 \leq MDR \leq 2$), or antagonistic (MDR < 0.5) effects [25]. To estimate the MDR, we calculated the toxic unit (TU) of each individual pesticide (FPF, PRO) and of the binary chemical mixture (FPF + PRO) [51].

We calculated the risk ratio (RR) and the risk difference (RD) to quantitatively express both relative (RR) and absolute (RD) size of the interactive effect of the chemical mixture on bee survival (frequency of dead bees; electronic supplementary material, table S3) and behaviour (frequency of abnormally behaving bees; electronic supplementary material, table S4) [65,66]. The RR was determined by dividing the observed effects by the expected effects and therefore cannot be calculated when the expected effect is 0 [65,66]. The RD is the difference between the ratio of observed and expected effects.

3. Results

(a) Pesticides synergistically increased mortality

The combination of FPF and PRO (FPF + PRO) synergistically increased mortality of both in-hive and forager bees (binomial proportion test, Holm correction; electronic supplementary material, table S3; figure 1*a*,*b*). The synergistic effect of FPF + PRO significantly reduced in-hive bee survival at 750 ng bee^{-1} (1–48 h after exposure, $RR_{Max} = 9$, $RD_{Max} = 44$; electronic supplementary material, table S3), 1500 ng bee^{-1} (1–48 h after exposure, $RR_{Max} = 5$, $RD_{Max} = 37$) and 3000 ng bee^{-1} of FPF (1 h after exposure, $RR_{1h} = 5$, $RD_{1h} = 23$). The synergistic effect of FPF + PRO significantly reduced forager survival at 750 ng bee^{-1} (1–48 h after exposure, $RR_{Max} = 5$, $RD_{Max} = 64$; electronic supplementary material, table S3), 1500 ng bee^{-1} (1 h after exposure, $RR_{Max} = 5$, $RD_{Max} = 27$) and 3000 ng bee^{-1} of FPF (1–2 h after exposure, $RR_{Max} = 11$, $RD_{Max} = 33$).

These synergistic effects were weaker at higher doses (electronic supplementary material, tables S3 and S4): FPF alone caused higher mortality at increasing doses, approaching the upper threshold of 100%, and thus reducing the difference between combined and individual treatments. Because synergy is better captured when the mortality of individual treatments is low, the synergistic effect was longer lasting for in-hive bees (i.e. 1–48 h at 1500 ng bee^{-1}), than for foragers (i.e. 1 h at 1500 ng bee^{-1}).

The LD_{50} of FPF + PRO ($TU_{FPF,summer\ in\text{-}hive} = 0.25$; $TU_{FPF,summer\ foragers} = 0.19$; $TU_{PRO} = 0.07$, $TU_{FPF+PRO,summer\ in\text{-}hive} = 0.32$, $TU_{FPF+PRO,\ summer\ foragers} = 0.26$) was also significantly lower than that of either compound alone for both in-hive (4-fold toxicity increase, MDR = 3.1) and foragers (5-fold toxicity increase, MDR = 3.9). Because the MDRs are higher than 2, the FPF + PRO interaction was synergistic for both worker types [64]. PRO alone did not cause any significant effect on survival ($\chi^2 = 0.6376$, d.f. = 1, $p > 0.42$).

The lower field-realistic FPF dose (375 ng FPF bee 1 h^{-1}) caused a 73% mortality in bees when combined with PRO. Thus, the FPF + PRO LD_{50} was lower than 375 ng FPF bee^{-1}.

There was a significant effect of FPF dose on bee survival (Fit proportional hazards, $p < 0.0001$; electronic supplementary material, table S5). Pesticide-free bees survived significantly longer than bees exposed to 1500 ng bee^{-1} (Kaplan–Meier^{DS}, $p < 0.049$; electronic supplementary material, table S6), 3000 ng bee^{-1} ($p < 0.0001$), 6000 ng bee^{-1} ($p < 0.0001$), 12 000 ng bee^{-1} ($p < 0.0001$) of FPF.

The FPF LD_{50} of our study (2995 ng bee^{-1}) is 2.5 times higher than the value reported by the US EPA (1200 ng bee^{-1}) [15] when we compare toxicity on standard individuals (in-hive summer bees [60], figure 2). However, we had a slightly different protocol than the US EPA [15]. We used 1 h exposure to 10 µl, not 6 h exposure *ad libitum*. Our positive control (DIM, reference toxin) met validity criteria because the 24 h LD_{50} of DIM was within the standard limits (0.10–0.35 µg bee^{-1}) defined by official international guidelines [60]. There was no significant effect of the colony on bee survival ($p > 0.05$; electronic supplementary material, table S5).

(b) Pesticides synergistically increased abnormal behaviours

FPF + PRO synergistically increased abnormal behaviours of both in-hive and forager bees (binomial proportion test, Holm correction; electronic supplementary material, table S4; figure 3). The synergistic effect of FPF + PRO significantly increased in-hive bee abnormal behaviours at the lower FPF doses of 750 ng bee^{-1} (1–4 h after exposure, $RR_{Max} = 10$, $RD_{Max} = 63$; electronic supplementary material, table S4), 1500 ng bee^{-1} (1–4 h after exposure, $RR_{Max} = 2$, $RD_{Max} = 33$)

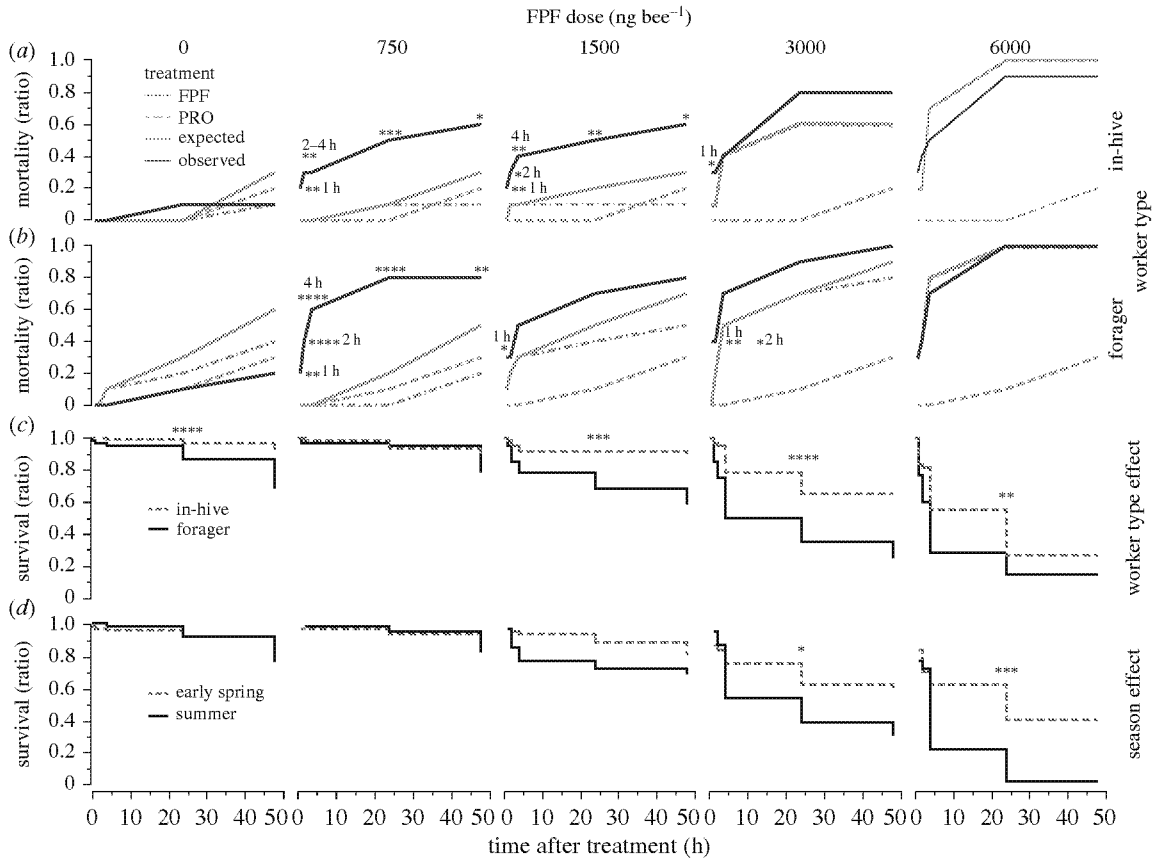

Figure 1. Lethal effects of FPF vary based on (*a*,*b*) combination with another pesticide (PRO), (*c*) bee worker type and (*d*) season. In (*a*,*b*), lethal synergistic effects of FPF + PRO on bee survival across time and worker type ((*a*) in-hive bees; (*b*) foragers). We tested the individual effects of FPF (blue dashed lines) and PRO (green dashed lines) and compared their expected (orange full lines) and observed (red full lines) combined effects, on bee mortality. In (*c*,*d*), we show the influence of (*c*) worker type and (*d*) season on bee sensitivity to FPF doses, assessed as survival across time. Asterisks indicate significant (*a*,*b*) synergistic (significant difference between mortality of expected and observed combined treatment; binomial proportion tests, Holm corrected; $n = 390$; electronic supplementary material, table S3) or (*c*,*d*) individual (Kaplan–Meier^{DS}; $n = 1440$; electronic supplementary material, table S6) effects of FPF at specific time assessments (*$p = 0.05$, **$p = 0.01$, ***$p = 0.001$, ****$p = 0.0001$).

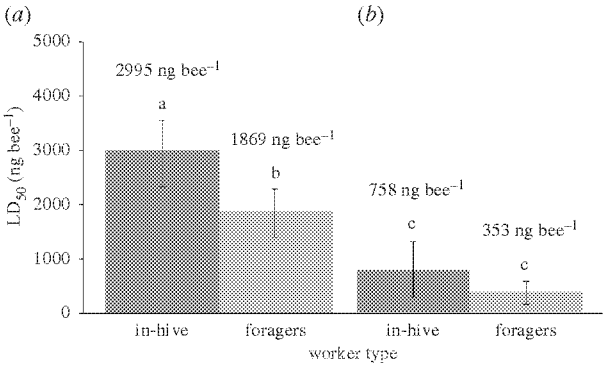

Figure 2. The LD_{50} (48 h) of bees exposed to FPF (*a*) and FPF + PRO (*b*) across worker types (in-hive bees versus foragers) in summer. Above each bar, we show the LD_{50} values. Different letters indicate significant differences. We show the 24 h LD_{50} of foragers (light grey bars), because high summer forager mortality at 48 h prevented the accurate estimation of their 48 h LD_{50} (standard LD_{50} estimation time, dark grey bars). Error bars represent 95% confidence intervals ($n_{overall} = 1080$). The LD_{50} of FPF across season and worker type is reported in electronic supplementary material, figure S1.

and 6000 ng bee^{-1} of FPF (4 h after exposure, $RR_{4h} = 2$, $RD_{4h} = 27$). The synergistic effect of FPF + PRO significantly increased forager abnormal behaviours at 750 ng bee^{-1} (1–4 h after exposure, $RR_{Max} = 15$, $RD_{Max} = 47$; electronic supplementary material, table S4), 1500 ng bee^{-1} (2–4 h after exposure, $RR_{Max} = 2$, $RD_{Max} = 30$) and 6000 ng bee^{-1} of FPF (1 h after exposure, $RR_{1h} = 2$, $RD_{1h} = 23$). PRO alone did not cause any significant abnormal behaviour. As with survival (see above), the synergistic effects on abnormal behaviours were more evident at lower doses.

The lower field-realistic dose of FPF (375 ng FPF bee 1 h^{-1}) significantly increased the number of bees exhibiting abnormal behaviours ($RR_{Max} = 36$, $RD_{Max} = 80$, $p < 0.0001$) when combined with PRO (electronic supplementary material, table S7; figure 3). These adverse behavioural effects started rapidly and remained consistent after treatment ($RR\ Range_{1-4h} = 34–36$, $RD_{1-4h} = 74–80$, $p < 0.0001$; table 1; electronic supplementary material, table S7).

There was a significant effect of dose 1, 2 and 4 h after treatment with FPF (Mixed $Model_{REML}$, $p < 0.0001$; electronic supplementary material, table S8; figure 3), DIM ($p < 0.0001$) and FPF + PRO ($p < 0.0001$). After treatment, bees showed coordination problems fairly consistent across time. Specifically, they mostly showed hyperactivity and curved-down abdomen in the shorter term after treatment (1 h) and apathy later on (4 h).

(c) Pesticides were more toxic to foragers than in-hive bees

There was a significant effect of worker type on the survival of bees exposed to FPF and DIM (fit proportional hazards,

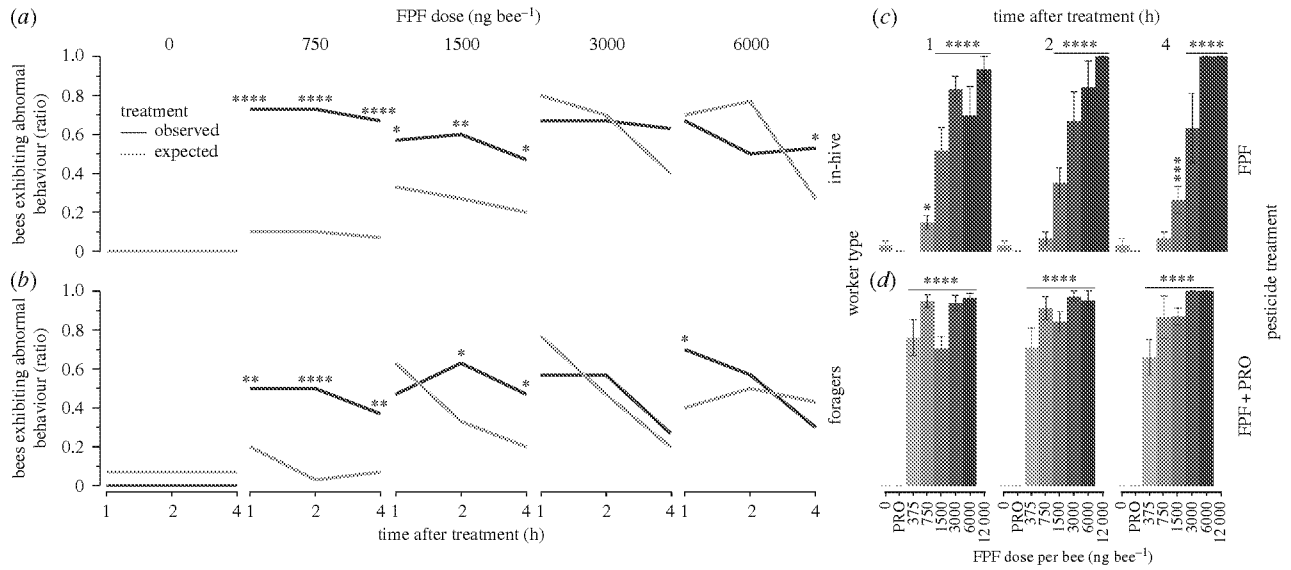

Figure 3. Sublethal (frequency of bees exhibiting abnormal behaviour) effects of FPF in combination with another pesticide (PRO). On the left (*a*,*b*), the synergistic effects of FPF and PRO across time (1–4 h after treatment) and worker type (in-hive bees: (*a*); foragers: (*b*)). We tested the individual effects of FPF and PRO and compared their expected (orange) and observed (red) combined effects (*a*,*b*, binomial proportion tests, Holm corrected; $n = 390$, electronic supplementary material, table S4). Because PRO alone did not alter bee behaviour alone, we only show the expected and observed results (*a*,*b*). On the right (*c*,*d*), sublethal effects of (*c*) FPF (750–12 000 ng FPF bee^{-1}) and (*d*) FPF + PRO (375–6000 ng FPF bee^{-1}, 7000 ng PRO bee^{-1}) as compared with each respective control (mixed model$_{REML}$, contrast test^{DS}, Dunn–Sidak corrected; $n = 342$; electronic supplementary material, tables S7 and S8). We show the effects observed in summer, pooled by worker type in (*c*,*d*). Asterisks indicate significant effects at specific time assessments (*$p = 0.05$, **$p = 0.01$, ****$p = 0.0001$).

Table 1. The frequency of bees exhibiting abnormal behaviours after exposure to the lower field-realistic FPF dose tested (375 ng FPF bee^{-1}), combined with PRO. We report the effect size as the risk ratio (RR, observed/expected) and the risk difference (RD, observed–expected), which compared the effects of FPF + PRO (375 ng FPF bee^{-1} and 7000 ng PRO bee^{-1}) versus either control (0 ng bee^{-1}) or the lower dose of FPF tested alone (750 ng FPF alone per bee). Combined exposure to FPF + PRO (375 ng FPF bee^{-1}) resulted in a higher frequency of bees exhibiting abnormal behaviours, even when compared with higher doses of FPF administered alone (750 ng bee^{-1}; $RR_{range} = 7–34$; $RD_{range} = 43–75$). Because of the low effect of FPF alone at 375 ng FPF bee^{-1}, we did not test the effects of this dose alone. We report 'n.a.' because the expected mortality was 0, and RR calculation is not possible when the denominator is 0.

treatment	worker type	time after treatment (h)	bees exhibiting abnormal behaviour (%)	synergistic increase			
				versus control		versus FPF alone	
				RR	RD	RR	RD
FPF + PRO (375 ng FPF bee^{-1})	in-hive	1	76	n.a.	76	34	66
		2	67	n.a.	67	30	56
		4	50	n.a.	50	22	43
	foragers	1	76	34	74	8	56
		2	74	33	72	7	71
		4	82	36	80	12	75

$p < 0.0001$; electronic supplementary material, table S5). Foragers are older than in-hive bees, and thus it is not surprising that in-hive control bees lived longer than control foragers (Kaplan–Meier^{DS}, $p = 0.0001$; electronic supplementary material, table S6; figure 1*c*). As the FPF dose increased, the survival of both bee castes decreased and, at each dose, the difference between the survival of each caste tended to increase (interaction FPF dose × worker type, $p = 0.001$; electronic supplementary material, table S5; figure 1*c*,*d*). FPF was significantly more toxic to foragers (compared with in-hive bees) at almost all doses tested: 1500 ng bee^{-1} (4-fold increase at 48 h, Kaplan–Meier^{DS}, $p = 0.0002$; electronic supplementary material, table S6), 3000 ng bee^{-1} (2-fold, $p < 0.0001$), 6000 ng bee^{-1} (1.2-fold, $p = 0.006$) of FPF. At 12 000 ng bee^{-1} of FPF, there was no significant effect ($p > 0.26$) of worker type on survival, because the mortality of in-hive and forager bees was very high.

The LD_{50} assessments confirmed that FPF and DIM toxicity was influenced by worker type (figure 2; electronic supplementary material, figure S2). Foragers were significantly more susceptible to pesticides in both early spring (FPF: 2-fold toxicity increase; DIM: 4-fold) and summer

(FPF: 2-fold; DIM: NS), as compared with in-hive bees. High forager mortality in summer prevented the estimation of the 48 h LD_{50} of summer foragers. Foragers were more affected by the synergistic effects caused by FPF + PRO (5-fold increased mortality), as compared with in-hive bees (4-fold).

There was a significant effect of worker type on bee abnormal behaviours 1 h after treatment with FPF (mixed $model_{REML}$, $p = 0.046$; electronic supplementary material, table S3), and 1 and 2 h after treatment with DIM ($p < 0.005$). There was no significant effect of worker type at any other time point ($p > 0.57$). There was no significant effect of worker type on abnormal behaviours after exposure to FPF + PRO ($p > 0.05$).

There was a significant effect of the interaction dose × worker type after treatment with FPF (1–2 h: $p < 0.049$), DIM (1–4 h: $p < 0.025$) and FPF + PRO (4 h: $p = 0.003$; electronic supplementary material, table S3; figure 3) on bee abnormal behaviours. Foragers were more susceptible to FPF (1500 ng bee^{-1}: $p < 0.0001$; electronic supplementary material, table S9) and FPF + PRO (375 ng bee^{-1}: $p < 0.006$; electronic supplementary material, table S9) as compared with in-hive bees.

(d) FPF was more toxic in summer

There was a significant effect of season on the survival of bees exposed to FPF (fit proportional hazards, $p < 0.0001$; electronic supplementary material, table S5). There was also a significant effect of the interaction FPF dose × season ($p = 0.011$). FPF was significantly more toxic in summer, as compared with early spring, at 3000 ng bee^{-1} (Kaplan–$Meier^{DS}$, $p = 0.013$; electronic supplementary material, table S6; figure 1*d*) and 6000 ng bee^{-1} ($p = 0.0003$). At 12 000 ng of FPF, there was no significant effect of season on survival, because bee mortality was too high in both seasons ($p > 0.78$).

The LD_{50} assessments confirmed that FPF toxicity was influenced by season (electronic supplementary material, figure S1). FPF was significantly more toxic in summer, both for in-hive and forager bees (2-fold toxicity increase), as compared with early spring.

There was a significant effect of season on bee abnormal behaviours 1 and 2 h after treatment with FPF (mixed $model_{REML}$, $p < 0.008$; electronic supplementary material, table S8). There was no significant effect of season at 4 h after treatment of FPF ($p > 0.51$). There was a significant effect of the interaction dose × season on bee abnormal behaviours after treatment with FPF (1 h: $p = 0.0004$; electronic supplementary material, table S8; figure S4). FPF significantly increased bee abnormal behaviours in summer, as compared with early spring, at 750 ng bee^{-1} (contrast $test^{DS}$, $p < 0.0001$; electronic supplementary material, table S9; figure S4), 1500 ng bee^{-1} ($p = 0.012$) and 3000 ng bee^{-1} ($p = 0.001$).

The electronic supplementary material contains additional results, including bee weight and DIM toxicity.

4. Discussion

We provide the first demonstration that the combination of two pesticides can synergistically increase the frequency of pollinators with abnormal behaviours (figure 3). We also provide the first evidence of adverse synergistic lethal ($MDR_{Max} = 4$; $RR_{Max} = 11$; $RD_{Max} = 64$) and sublethal ($RR_{Max} = 15$; $RD_{Max} = 63$) effects caused by FPF and an SBI fungicide, PRO (electronic supplementary material, tables S3 and S4; figures 1 and 3). All FPF doses tested significantly impaired bee behaviour as compared with the control treatment (electronic supplementary material, tables S7 and S8; figure 3). FPF can thus impair bee survival and behaviour at field-realistic (worst-case) doses when combined with an SBI fungicide. In addition, the toxic effect of FPF and FPF + PRO on bee survival and behaviour was significantly influenced by worker type and season. Foragers were consistently more susceptible to these pesticides (up to 4-fold; figures 1 and 3; electronic supplementary material, figures S2–S4). This result is troubling because the official guidelines for pesticide risk assessment (RA) only test in-hive bees, thereby underestimating the risk that pesticides pose for foragers. This is also concerning given that foragers are particularly at risk of pesticide exposure since they forage in the field. The lower weight of foragers (−11%), as compared with in-hive bees (electronic supplementary material, table S10), is a possible reason for their increased susceptibility to pesticides.

Our results confirm that abnormal behaviours usually appear shortly (1 h) after exposure to pesticides [40,67,68]. However, official RA guidelines do not require a thorough assessment of abnormal behaviours, and then only 4 h after treatment, when many effects may have declined. Short-term (1–2 h) behavioural alterations can be detrimental for bee health, especially for bees carrying out risky tasks (i.e. foraging) within this time window. We demonstrated that adverse sublethal effects are more frequent in foragers (as compared with in-hive bees) and could impact their foraging efficiency, as well as their survival, if these abnormal behaviours occur while bees are foraging in the field. Future behavioural assessments conducted using standard assays such as locomotion arenas could better establish potentially harmful effects of pesticides on beneficial insects [67,69–71].

Our results show that FPF and the neonicotinoids, both of which target insect nicotinic acetylcholine receptors [9], have relatively similar effects on bee health, sharing side-effects on bee survival and behaviour [67]. The SBI fungicide, PRO, similarly amplifies both FPF (4-fold, $MDR_{summer\ in\text{-}hive} = 3.1$, this study) and neonicotinoid (3-fold [22]) toxicity in bees. With respect to survival, FPF toxicity (based on LD_{50}) is over 559 times lower than the toxicity of the *N*-nitroguanidine neonicotinoids (clothianidin, imidacloprid, thiamethoxam), but more than five times higher than the *N*-cyanoamidine neonicotinoids (acetamiprid, thiacloprid) [33]. To assess the risk for bees, these differences need to be considered based on actual exposure, which depends on application methods (e.g. frequency of treatments and application rate) and active ingredient properties (e.g. toxicity).

The FPF + PRO synergistic effects on bee survival and behaviour are more evident at lower doses, where the effect of FPF is less detrimental. Our assessment suggests that FPF, like the neonicotinoids [40], may lead to a favourable biological response at low exposure levels (i.e. hormesis [72]). In foragers, the low dose of FPF (750 ng bee^{-1}) resulted in reduced mortality than the control treatment (0 ng bee^{-1}; figure 1*c*,*d*). Similarly, our observed synergistic behavioural alterations (figure 3) occurred at low (750–1500 ng bee^{-1}) and high (6000 ng bee^{-1}) doses, but not at intermediate ones (3000 ng bee^{-1}). These non-monotonic effects of pesticides (i.e. hormesis) should be further investigated.

Restrictions on neonicotinoids have been increasing after decades of research demonstrating their adverse effects on beneficial pollinators and persistent environmental contamination [20,58]. New systemic insecticides such as FPF and sulfoxaflor, examples of the novel butenolide and sulfoxamine chemical classes, are the likely successors of the neonicotinoids [73]. While sulfoxaflor has adverse effects on bumblebees [74], FPF is considered 'bee safe' and can be used on flowering crops with actively foraging bees [12,15]. Our study raises concerns about the ecotoxicological profile of FPF and the safety of FPF for bees.

Pesticide toxicity is amplified by multiple interacting stressors [5,20] and thus a holistic approach that tests more of the different conditions that bees naturally experience is beneficial [39,75]. Although sensitivity to pesticides is influenced by season, bee type and concomitant pesticide exposure [44,76], current RA schemes implement only limited tests, requiring only the evaluation of single pesticide effects on in-hive summer bees [77]. Such restricted testing could underestimate or overestimate pesticide effects [38,77] because different worker types (i.e. in-hive bees versus foragers) can be exposed to multiple pesticides over different seasons [6]. FPF (Sivanto) labelling restricts using tank-mixtures with azole fungicides [10]. However, bees can still be exposed to FPF and PRO simultaneously, as discussed above and in the electronic supplementary material.

Future RA should consider sublethal and behavioural effects [78,79]. Studies are necessary to confirm and link laboratory results to field tests, so that specific protection goals, such as avoiding unacceptable decreases in colony population or increases in forager mortality, can be assessed [78]. Monitoring the behaviour of multiple honeybees in a whole colony, though feasible in observation colonies [80], introduces complications [19] and costs that would be likely to constrain general adoption in RA. Testing bees in the laboratory also has the benefit of greater control, ease of testing and thus replication with more colony sources.

Because RA with honeybees is often used as an indicator of potential harm to other bees [50], our results raise broader concerns. However, there can be significant differences, greater and lesser, between the individual sensitivities of different bee species, highlighting the need for future research.

Although risk assessors are beginning to address synergistic effects on survival [79,81] and sublethal effects such as homing behaviour and hypopharyngeal development [51], many important synergistic and behavioural effects that can affect colony fitness are not explored. We propose that RA include more thorough assessments of behaviours and synergies. Behavioural testing should be implemented 1 or 2 h after exposure, since short-term behavioural effects can realistically impair bee survival when ingesting pesticides while foraging [82]. RA could address potential synergies on behaviour and survival by testing limited chemical mixtures of pesticides that have a higher likelihood of interactive effects based on the respective modes of action (e.g. propiconazole with neonicotinoids or FPF) and co-occur in the environment. We provide a simple way to measure synergistic effects on bee behaviour and survival following a standard ecotoxicological test. Our procedure could be implemented fairly easily in pesticide RA procedures, within the LD_{50} toxicity test scheme [60].

Data accessibility. Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.5f87k5v [83].

Authors' contributions. S.T. and J.C.N. designed and supervised the experiments, analysed the data and wrote the manuscript.

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References

1. Klein A-M, Vaissiere BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, Tscharntke T. 2007 Importance of pollinators in changing landscapes for world crops. *Proc. Biol. Sci.* **274**, 303–313. (doi:10.1098/rspb.2006.3721)
2. Hung K-LJ, Kingston JM, Albrecht M, Holway DA, Kohn JR. 2018 The worldwide importance of honey bees as pollinators in natural habitats. *Proc. R. Soc. B* **285**, 20172140. (doi:10.1098/rspb.2017.2140)
3. Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O, Kunin WE. 2010 Global pollinator declines: trends, impacts and drivers. *Trends Ecol. Evol.* **25**, 345–353. (doi:10.1016/j.tree.2010.01.007)
4. Desneux N, Decourtye A, Delpuech J-MM. 2007 The sublethal effects of pesticides on beneficial arthropods. *Annu. Rev. Entomol.* **52**, 81–106. (doi:10.1146/annurev.ento.52.110405.091440)
5. Goulson D, Nicholls E, Botías C, Rotheray EL. 2015 Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* **347**, 1255957. (doi:10.1126/science.1255957)
6. Tosi S, Costa C, Vesco U, Quaglia G, Guido G. 2018 A 3-year survey of Italian honey bee-collected pollen reveals widespread contamination by agricultural pesticides. *Sci. Total Environ.* **615**, 208–218. (doi:10.1016/j.scitotenv.2017.09.226)
7. Stokstad E. 2017 European bee study fuels debate over pesticide ban. *Science* **356**, 1321. (doi:10.1126/science.356.6345.1321)
8. Butler D. 2018 Scientists hail European ban on bee-harming pesticides. *Nature* (27 April). (doi:10.1038/d41586-018-04987-4)
9. Giorio C, Safer A, Sánchez-Bayo F, Tapparo A, Lentola A, Girolami V, Bijleveld van Lexmond M, Bonmatin J-M. 2017 An update of the Worldwide Integrated Assessment (WIA) on systemic insecticides. Part 1: new molecules, metabolism, fate, and transport. *Environ. Sci. Pollut. Res.* (doi:10.1007/s11356-017-0394-3)
10. Bayer Crop Science AG. 2013 *Flupyradifurone technical information*. Monheim am Rhein, Germany: Bayer Crop Science AG.
11. Jeschke P, Nauen R, Gutbrod O, Beck ME, Matthiesen S, Haas M, Velten R. 2015 Flupyradifurone (Sivanto™) and its novel butenolide pharmacophore: structural considerations. *Pestic. Biochem. Physiol.* **121**, 31–38. (doi:10.1016/j.pestbp.2014.10.011)
12. Nauen R *et al.* 2014 Flupyradifurone: a brief profile of a new butenolide insecticide. *Pest Manag. Sci.* **71**, 850–862. (doi:10.1002/ps.3932)
13. Malev O, Klobučar RS, Fabbretti E, Trebše P. 2012 Comparative toxicity of imidacloprid and its transformation product 6-chloronicotinic acid to non-target aquatic organisms: microalgae *Desmodesmus subspicatus* and amphipod *Gammarus*

fossarum. *Pestic. Biochem. Physiol.* **104**, 178–186. (doi:10.1016/j.pestbp.2012.07.008)

14. Prosser RS, de Solla SR, Holman EAM, Osborne R, Robinson SA, Bartlett AJ, Maisonneuve FJ, Gillis PL. 2016 Sensitivity of the early-life stages of freshwater mollusks to neonicotinoid and butenolide insecticides. *Environ. Pollut.* **218**, 428–435. (doi:10.1016/j.envpol.2016.07.022)
15. US EPA. 2014 *Environmental fate and ecological risk assessment for foliar, soil drench, and seed treatment uses of the new insecticide flupyradifurone (BYI 02960)*. Washington, DC: US EPA.
16. Hesselbach H, Scheiner R. 2018 Supplementary information of effects of the novel pesticide flupyradifurone (Sivanto) on honeybee taste and cognition. *Sci. Rep.* **8**, 4954. (doi:10.1038/s41598-018-23200-0)
17. Tan K, Chen W, Dong S, Liu X, Wang Y, Nieh JC. 2015 A neonicotinoid impairs olfactory learning in Asian honey bees (*Apis cerana*) exposed as larvae or as adults. *Sci. Rep.* **5**, 2–10. (doi:10.1038/srep10989)
18. Campbell JW, Cabrera AR, Stanley-Stahr C, Ellis JD. 2016 An evaluation of the honey bee (Hymenoptera: Apidae) safety profile of a new systemic insecticide, flupyradifurone, under field conditions in Florida. *J. Econ. Entomol.* **96**, 875–878. (doi:10.1093/jee/tow186)
19. Simon-Delso N, San Martin G, Bruneau E, Delcourt C, Hautier L. 2017 The challenges of predicting pesticide exposure of honey bees at landscape level. *Sci. Rep.* **7**, 3801. (doi:10.1038/s41598-017-03467-5)
20. Tosi S, Nieh JC, Sgolastra F, Cabbri R, Medrzycki P. 2017 Neonicotinoid pesticides and nutritional stress synergistically reduce survival in honey bees. *Proc. R. Soc. B* **284**, 20171711. (doi:10.1098/rspb.2017.1711)
21. Alaux C *et al.* 2010 Interactions between *Nosema* microspores and a neonicotinoid weaken honeybees (*Apis mellifera*). *Environ. Microbiol.* **12**, 774–782. (doi:10.1111/j.1462-2920.2009.02123.x)
22. Sgolastra F *et al.* 2017 Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species. *Pest Manag. Sci.* **73**, 1236–1243. (doi:10.1002/ps.4449)
23. Yao J, Zhu YC, Adamczyk J. 2018 Responses of honey bees to lethal and sublethal doses of formulated clothianidin alone and mixtures. *J. Econ. Entomol.* **20**, 1–9. (doi:10.1093/jee/toy140)
24. Prado A, Pioz M, Vidau C, Requier F, Jury M, Crauser D, Brunet J, Le Y, Alaux C. 2019 Exposure to pollen-bound pesticide mixtures induces longer-lived but less efficient honey bees. *Sci. Total Environ.* **650**, 1250–1260. (doi:10.1016/j.scitotenv.2018.09.102)
25. Belden JB, Gilliom RJ, Lydy MJ. 2007 How well can we predict the toxicity of pesticide mixtures to aquatic life? *Integr. Environ. Assess. Manag.* **3**, 364–372. (doi:10.1897/1551-3793(2007)3[364:HWCWPT]2.0.CO;2)
26. Pérez J, Domingues I, Monteiro M, Soares AMVM, Loureiro S. 2013 Synergistic effects caused by atrazine and terbuthylazine on chlorpyrifos toxicity to early-life stages of the zebrafish *Danio rerio*. *Environ. Sci. Pollut. Res.* **20**, 4671–4680. (doi:10.1007/s11356-012-1443-6)
27. Quintela ED, McCoy CW. 1998 Synergistic effect of imidacloprid two entomopathogenic fungi on the behavior and survival of larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in soil. *Biol. Microb. Control* **91**, 110–122. (doi:10.1093/jee/91.1.110)
28. Iwasa T, Motoyama N, Ambrose JT, Roe RMM. 2004 Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Prot.* **23**, 371–378. (doi:10.1016/j.cropro.2003.08.018)
29. Traynor KS, Pettis JS, Tarpy DR, Mullin CA, Frazier JL, Frazier M, Vanengelsdorp D. 2016 In-hive pesticide exposome: assessing risks to migratory honey bees from in-hive pesticide contamination in the Eastern United States. *Sci. Rep.* **6**, 1–16. (doi:10.1038/srep33207)
30. Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R, VanEngelsdorp D, Pettis JS. 2010 High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. *PLoS ONE* **5**, e9754. (doi:10.1371/journal.pone.0009754)
31. Battaglin WA, Sandstrom MW, Kuivila KM, Kolpin DW, Meyer MT. 2011 Occurrence of azoxystrobin, propiconazole, and selected other fungicides in US streams, 2005–2006. *Water. Air. Soil Pollut.* **218**, 307–322. (doi:10.1007/s11270-010-0643-2)
32. Hladik ML, Vandever M, Smalling KL. 2016 Exposure of native bees foraging in an agricultural landscape to current-use pesticides. *Sci. Total Environ.* **542**, 469–477. (doi:10.1016/j.scitotenv.2015.10.077)
33. Lewis KA, Tzilivakis J, Warner DJ, Green A. 2016 An international database for pesticide risk assessments and management. *Hum. Ecol. Risk Assess.* **22**, 1050–1064. (doi:10.1080/10807039.2015.1133242)
34. Wahl O, Ulm K. 1983 Influence of pollen feeding and physiological condition on pesticide sensitivity of the honey bee *Apis mellifera carnica*. *Oecologia* **59**, 106–128. (doi:10.1007/BF00388082)
35. Decourtye A, Lacassie E, Pham-Delegue M-H. 2003 Learning performances of honeybees (*Apis mellifera* L) are differentially affected by imidacloprid according to the season. *Pest Manag. Sci.* **59**, 269–278. (doi:10.1002/ps.631)
36. Meled M, Thrasyvoulou A, Belzunces LP. 1998 Seasonal variations in susceptibility of *Apis mellifera* to the synergistic action of prochloraz and deltamethrin. *Environ. Toxicol. Chem.* **17**, 2517–2520. (doi:10.1002/etc.5620171220)
37. Smirle MJ, Winston ML. 1987 Intercolony variation in pesticide detoxification by the honey bee (Hymenoptera: Apidae). *J. Econ. Entomol.* **80**, 5–8. (doi:10.1093/jee/80.1.5)
38. Baines D, Wilton E, Pawluk A, De Gorter M, Chomistek N. 2017 Neonicotinoids act like endocrine disrupting chemicals in newly-emerged bees and winter bees. *Sci. Rep.* **7**, 10979. (doi:10.1038/s41598-017-10489-6)
39. Poquet Y, Vidau C, Alaux C. 2016 Modulation of pesticide response in honeybees. *Apidologie* **47**, 412–426. (doi:10.1007/s13592-016-0429-7)
40. Tosi S, Démares FJ, Nicolson SW, Medrzycki P, Pirk CWW, Human H. 2016 Effects of a neonicotinoid pesticide on thermoregulation of African honey bees (*Apis mellifera* scutellata). *J. Insect Physiol.* **93–94**, 56–63. (doi:10.1016/j.jinsphys.2016.08.010)
41. Gerig L. 1975 The effects of juvenile hormone analogues on summer bees (*Apis mellifera* L.) in the field and laboratory. *Schweiz. Landwirtsch. Forsch* **14**, 355–370.
42. Tahori AS, Sobel Z, Soller M. 1969 Variability in insecticide tolerance of eighteen honey-bee colonies. *Entomol. Exp. Appl.* **12**, 85–98. (doi:10.1111/j.1570-7458.1969.tb02501.x)
43. Vance JT, Williams JB, Elekonich MM, Roberts SP. 2009 The effects of age and behavioral development on honey bee (*Apis mellifera*) flight performance. *J. Exp. Biol.* **212**, 2604–2611. (doi:10.1242/jeb.028100)
44. Harris JW, Woodring J. 1992 Effects of stress, age, season, and source colony on levels of octapamine, dopamine and serotonin in the honeybee (*Apis mellifera* L.) brain. *J. Insect Physiol.* **38**, 29–35. (doi:10.1016/0022-1910(92)90019-A)
45. Ladas A. 1972 The influence of some internal and external factors upon the insecticide resistance of honeybees. *Apidologie* **3**, 55–78. (doi:10.1051/apido:19720103)
46. Guez D, Suchail S, Gauthier M, Maleszka R, Belzunces LP. 2001 Contrasting effects of imidacloprid on habituation in 7- and 8-day-old honeybees (*Apis mellifera*). *Neurobiol. Learn. Mem.* **76**, 183–191. (doi:10.1006/nlme.2000.3995)
47. Mayland PG, Burkhardt CC. 1970 Honey bee mortality as related to insecticide-treated surfaces and bee age. *J. Econ. Entomol.* **63**, 1437–1439. (doi:10.1093/jee/63.5.1437)
48. Rinkevich FD, Margotta JW, Pittman JM, Danka RG, Tarver MR, Ottea JA, Healy KB. 2015 Genetics, synergists, and age affect insecticide sensitivity of the honey bee, *Apis mellifera*. *PLoS ONE* **10**, e0139841. (doi:10.1371/journal.pone.0139841)
49. Williams GR *et al.* 2013 Standard methods for maintaining adult *Apis mellifera* in cages under *in vitro* laboratory conditions. *J. Apic. Res.* **52**, 1–36. (doi:10.3896/IBRA.1.52.1.04)
50. US EPA. 2012 *White paper in support of the proposed risk assessment process for bees*. Washington, DC: US EPA.
51. EFSA. 2013 EFSA Guidance Document on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). *EFSA J.* **11**, 268. (doi:10.2903/j.efsa.2013.3295)
52. Thompson HM, Fryday SL, Harkin S, Milner S. 2014 Potential impacts of synergism in honeybees (*Apis mellifera*) of exposure to neonicotinoids and sprayed fungicides in crops. *Apidologie* **45**, 545–553. (doi:10.1007/s13592-014-0273-6)
53. Syngenta. 2009 *QuiltXcel—broad spectrum fungicide for control of plant diseases*. Basel, Switzerland: Syngenta.

54. US EPA. 2017 *Propiconazole EC*. Washington, DC: US EPA.
55. Knezevic S. 2017 *2017 guide for weed, disease, and insect management in Nebraska*. Lincoln, NE: Board of Regents of the University of Nebraska–Lincoln.
56. Botías C, David A, Hill EM, Goulson D. 2016 Contamination of wild plants near neonicotinoid seed-treated crops, and implications for non-target insects. *Sci. Total Environ.* **566–567**, 269–278. (doi:10.1016/j.scitotenv.2016.05.065)
57. David A, Botías C, Abdul-Sada A, Nicholls E, Rotheray EL, Hill EM, Goulson D. 2016 Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops. *Environ. Int.* **88**, 169–178. (doi:10.1016/j.envint.2015.12.011)
58. Bonmatin J-M *et al*. 2014 Environmental fate and exposure; neonicotinoids and fipronil. *Environ. Sci. Pollut. Res. Int.* **22**, 35–67. (doi:10.1007/s11356-014-3332-7)
59. Woodcock BA *et al*. 2018 Neonicotinoid residues in UK honey despite European Union moratorium. *PLoS ONE* **13**, 1–15. (doi:10.1371/journal.pone.0189681)
60. OECD/OCDE. 1998 OECD Guideline 213 for the testing of chemicals: honeybees, acute oral toxicity test. (doi:10.1787/9789264070165-en)
61. Medrzycki P *et al*. 2013 Standard methods for toxicology research in *Apis mellifera*. *J. Apic. Res.* **52**, 1–60. (doi:10.3896/IBRA.1.52.4.14)
62. Cox RL, Wilson WT. 1984 Effects of permethrin on the behavior of individually tagged honey bees, *Apis mellifera* L. (Hymenoptera: Apidae). *Env. Entomol.* **13**, 375–378. (doi:10.1093/ee/13.2.375)
63. OECD. 2017 *OECD guideline 245 for the testing of chemicals. Honey bee* (Apis mellifera *L.*), *chronic oral toxicity test (10-day feeding)*. Paris, France: OECD. (doi:10.1787/9789264284081-en)
64. Cedergreen N. 2014 Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology. *PLoS ONE* **9**, e96580. (doi:10.1371/journal.pone.0096580)
65. Deeks JJ, Higgins JP, Altman DG. 2011 Effect measures for dichotomous outcomes. In *Cochrane handbook for systematic reviews of interventions* (eds J Higgins, S Green). Chichester, UK: John Wiley.
66. Noordhuizen J, Frankena K, Thrusfield M, Graat E. 2001 *Application of quantitative methods in veterinary epidemiology*. Wageningen, The Netherlands: Wageningen Pers.
67. Tosi S, Nieh JC. 2017 A common neonicotinoid pesticide, thiamethoxam, alters honey bee activity, motor functions, and movement to light. *Sci. Rep.* **7**, 15132. (doi:10.1038/s41598-017-15308-6)
68. Tosi S, Burgio G, Nieh JCJC. 2017 A common neonicotinoid pesticide, thiamethoxam, impairs honey bee flight ability. *Sci. Rep.* **7**, 1201. (doi:10.1038/s41598-017-01361-8)
69. Charreton M, Decourtye A, Henry M, Rodet G, Sandoz J-C, Charnet P, Collet C. 2015 A locomotor deficit induced by sublethal doses of pyrethroid and neonicotinoid insecticides in the honeybee *Apis mellifera*. *PLoS ONE* **10**, e0144879. (doi:10.1371/journal.pone.0144879)
70. El Hassani AK, Dacher M, Gary V, Lambin M, Gauthier M, Armengaud C, El Hassani A, Dacher M, Gary V. 2008 Effects of sublethal doses of acetamiprid and thiamethoxam on the behavior of the honeybee (*Apis mellifera*). *Arch. Environ. Contam. Toxicol.* **54**, 653–661. (doi:10.1007/s00244-007-9071-8)
71. Maze IS, Wright GA, Mustard JA. 2006 Acute ethanol ingestion produces dose-dependent effects on motor behavior in the honey bee (*Apis mellifera*). *J. Insect Physiol.* **52**, 1243–1253. (doi:10.1016/j.jinsphys.2006.09.006)
72. Tang Q, Ma K, Chi H, Hou Y, Id XG. 2019 Transgenerational hormetic effects of sublethal dose of flupyradifurone on the green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). *PLoS ONE*, **14**, e0208058. (doi:10.1371/journal.pone.0208058)
73. Brown MJF *et al*. 2016 A horizon scan of future threats and opportunities for pollinators and pollination. *PeerJ* **4**, e2249. (doi:10.7717/peerj.2249)
74. Siviter H, Brown MJF, Leadbeater E. 2018 Sulfoxaflor exposure reduces bumblebee reproductive success. *Nature* **561**, 109. (doi:10.1038/s41586-018-0430-6)
75. EFSA. 2016 Assessing the health status of managed honeybee colonies (HEALTHY-B): a toolbox to facilitate harmonised data collection. *EFSA J.* **14**, 4578. (doi:10.2903/j.efsa.2016.4578)
76. Castillo C, Maisonnasse A, Conte Y Le, Plettner E. 2012 Seasonal variation in the titers and biosynthesis of the primer pheromone ethyl oleate in honey bees. *J. Insect Physiol.* **58**, 1112–1121. (doi:10.1016/j.jinsphys.2012.05.010)
77. Decourtye A, Henry M, Desneux N. 2013 Overhaul pesticide testing on bees. *Nature* **497**, 188. (doi:10.1038/497188a)
78. Rortais A, Arnold G, Dorne J-L, More SJ, Sperandio G, Streissl F, Szentes C, Verdonck F. 2017 Risk assessment of pesticides and other stressors in bees: principles, data gaps and perspectives from the European Food Safety Authority. *Sci. Total Environ.* **587–588**, 524–537. (doi:10.1016/j.scitotenv.2016.09.127)
79. EFSA. 2012 Scientific opinion on the science behind the development of a risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). *EFSA J.* **10**, 1–275. (doi:10.2903/j.efsa.2012.2668)
80. Gernat T, Rao VD, Middendorf M, Dankowicz H, Goldenfeld N, Robinson GE, Holme P, Naug D, Sokolowski MB. 2018 Automated monitoring of behavior reveals bursty interaction patterns and rapid spreading dynamics in honeybee social networks. *Proc. Natl Acad. Sci. USA* **115**, 1–6. (doi:10.1073/pnas.1713568115)
81. Spurgeon D, Hesketh H, Lahive E, Svendsen C, Baas J, Robinson A, Horton A, Heard M. 2016 Chronic oral lethal and sub-lethal toxicities of different binary mixtures of pesticides and contaminants in bees (*Apis mellifera*, *Osmia bicornis* and *Bombus terrestris*). *EFSA Support. Publ.* **13**, EN-1076. (doi:10.2903/SP.EFSA.2016.EN-1076)
82. Henry M, Cerrutti N, Aupinel P, Decourtye A, Gayrard M, Odoux J-F, Pissard A, Rüger C, Bretagnolle V. 2015 Reconciling laboratory and field assessments of neonicotinoid toxicity to honeybees. *Proc. R. Soc. B* **282**, 20152110. (doi:10.1098/rspb.2015.2110)
83. Tosi S, Nieh JC. 2019 Data from: Lethal and sublethal synergistic effects of a new systemic pesticide, flupyradifurone (Sivanto®) on honeybees. Dryad Digital Repository. (doi:10.5061/dryad.5f87k5v)

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Lethal and sublethal synergistic effects of a new systemic pesticide, flupyradifurone (Sivanto®) on honey bees

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ESM Methods

This study was conducted at University of California San Diego (UCSD), Division of Biological Sciences (La Jolla, CA, USA). We used six healthy honey bee colonies (*Apis mellifera ligustica* Spinola, 1806, 10 frames per colony [1–3]) housed at the UCSD Biology Field Station apiary. In total, we recorded the survival and abnormal behaviours of 1860 bees and the weight of 354 bees. All assessments were conducted by experimenters blind to the treatments. We followed standard toxicological procedures for pesticide tests on bees [4,5].

Honey bee preparation

Foragers are the bees most likely exposed to flupyradifurone (FPF) since they can directly collect pollen and nectar from treated crops. In-hive bees can also be exposed to FPF because they receive the nectar and pollen collected by the foragers and consume potentially contaminated honey and pollen stores. We captured individually returning pollen foragers at hive entrances in vials. We caught in-hive bees located in combs with brood inside the colonies using standard procedures: these bees were likely nurses [6]. The difference between in-hive and forager bees was additionally confirmed by our weight results (see Results). After collection, in-hive and forager bees were separately placed into plastic cages (11 x 11 x 9 cm, 10 bees/cage) and maintained in an incubator at 25 ± 1°C and 50-80% RH for 72 h [5]. To facilitate consumption of the test solution, the bees were starved for 1 h before feeding [5,6].

Pesticide concentrations and doses

FPF (4D IRAC subgroup) is a newly developed systemic insecticide [7] that was first marketed in 2014 in Guatemala and Honduras [8], then in 2015 in USA [9], EU [10], and other countries [11]. Because FPF is a relatively recent pesticide, there is limited environmental contamination data available [12,13]. Nonetheless, it can be used on diverse crops (vegetables, potatoes, pome fruits, grapes, citrus, cotton, soybean, coffee, cocoa, hops, and ornamentals) though multiple application methods (spray, drip irrigation, soil treatments, and seed treatments) [8,14].

Because foragers mainly consume nectar, we calculated the field-realistic exposure of foragers based on the residue of FPF found in nectar (a realistic carbohydrate source) collected

by a forager, as sampled from its honey stomach [13]. In-hive bees consume nectar and pollen (realistic diet containing carbohydrates and proteins), and we therefore considered nectar and pollen contamination when estimating in-hive bee pesticide intake [15,16].

FPF was found at 4.3 ppm and 4.1 ppm in the nectar in the honey stomachs of bees that were foraging on oilseed rape crops respectively 1 and 3 days after FPF spray treatment (US EPA, 2014). Pollen collected by bees foraging on oilseed rape fields contained 21 ppm of FPF [13]. We simulated a worst-case scenario in which bees were foraging on oilseed rape crops, and therefore used FPF residues in nectar (4.3 ppm) and pollen (21 ppm) of oilseed rape. However, bees can be exposed to FPF at even higher concentrations, and for longer periods. Bees ingested FPF when collecting nectar from cotton (22 ppm) or pollen from apple (39 ppm) or blueberry (68 ppm) crops [13]. FPF was found for about 3 weeks in nectar collected by foragers, and up to nearly 5 months after initial exposure in the nectar and honey stored inside bee colonies [13].

With respect to dosages, we calculated the worst-case field-realistic FPF oral exposure levels for bees using European Food Safety Authority (EFSA) and Environmental Protection Agency (EPA) methods. Using these methods, we estimate that foragers collecting nectar in a field previously treated with FPF can be exposed to 550 ng FPF/bee per foraging flight, and up to 5504 ng FPF/bee per foraging day. These calculations were based on EFSA [16] and used the highest field-realistic FPF concentration found in the honey stomachs of bees that were foraging on oilseed rape crops, 4.3 ppm [13], and the sugar concentration of oilseed rape nectar (10% w/w [17,18]). Bees can be exposed to up to 1564 ng FPF/bee/foraging flight when they forage on nectar from cotton fields. These calculations were based on EFSA [16] and used the highest field-realistic FPF concentration found in the honey stomachs of bees that were foraging on cotton crops, 22 ppm [13], and a low field-realistic sugar concentration of cotton nectar (18%, [19]). Unlike foragers, nurses ingest less nectar and more pollen, leading to a field-realistic exposure of 2402 ng FPF/bee/day [16]. This calculation was based on EFSA guidelines [16] and considered intake of FPF contaminated pollen using the highest field-realistic empirical FPF concentration in oilseed rape pollen (21 ppm [13]). According to other calculations [15], the refined Estimated Environmental Concentration (EEC) of FPF is 970 ng/bee and 1256 ng/bee for nurses and foragers, respectively, when colonies forage in oilseed rape crops [13]. When bees forage nectar in cotton fields, refined EEC for workers reaches 6370 ng FPF/bee [13]. Thus, we used a FPF dose of 375 ng/bee that was lower than the field-realistic scenario in which bees

ingested FPF contaminated oilseed nectar during a single foraging flight. The tested FPF dose of 750 ng/bee was less than the field-realistic scenario in which bees ingested contaminated oilseed nectar for 1 day or cotton nectar for a foraging flight. Because of the limited FPF residue data availability, these estimations were based on *ad hoc* trials performed for pesticide registration purposes, before product authorisation. Thus, the estimations of FPF field-realistic doses and concentrations should be updated with data collected monitoring real field use in multiple scenarios.

FPF and propiconazole (PRO) are used in a variety of vegetable, fruit, and ornamental plants that are visited by bees. Because FPF is a new pesticide, no monitoring studies have yet tested its co-occurrence as a contaminant with other pesticides. However, FPF and neonicotinoids are approved for use on many of the same crops [8,13,14,20–22], and pollen collected by bees contained both neonicotinoids and SBI (Sterol Biosynthesis Inhibitor) fungicides [23]. Further screening of environmental contamination following large-scale real-world use after widespread commercialization, and testing of lower and field-realistic doses of FPF and PRO, are crucial to appropriately assess real and frequent risks caused by the pesticides [24]. The US EPA [13] assessed the combined effect of FPF and the fungicide, tebuconazole, and found that the addition of tebuconazole (ratio 1:7.5, respectively) decreased FPF LD_{50} of in-hive bees by 6 fold (1200 ng FPF/bee vs. 200 ng FPF+tebuconazole/bee). Although risk assessors are developing models to predict multiple chemical interactions based on chemical characteristics, ultimately reducing the amount of laboratory trials needed to assess risk, the data available is still poor and ultimately require empirical validation [25,26].

The acute oral dose-response relationship (i.e. LD_{50} test) was evaluated using five doses in a geometric series, with a common ratio factor of 2 ($FPF_{LD50\ dose\ range}$: 750-12000 ng/bee) [5]. Because of the high mortality of the FPF+PRO treatments, we tested an additional lower dose of 375 ng FPF/bee (corresponding to 37.5 ppm), instead of the higher 12000 ng FPF/bee, for the combined treatment. In table 1, we compare FPF+PRO treatments that used 375 ng FPF/bee with a higher dose of FPF alone (750 ng/bee), but our estimate of synergism is likely conservative: as expected 750 ng FPF/bee led to stronger effects than 375 ng FPF/bee (data from preliminary test). Because of high summer mortality, we tested an additional lower dose of DIM (25 ng DIM/bee, corresponding to 25 ppm), instead of the higher 800 ng DIM/bee. The synergistic

effects of FPF+PRO were only tested in the summer, since this season is the most standard one for testing toxicity [5].

We used analytical grade FPF (CAS# 951659-40-8, PESTANAL® analytical standard, Sigma-Aldrich, purity: 99.9%), DIM (CAS# 60-51-5, PESTANAL® analytical standard, Sigma-Aldrich, purity: 99.5%), and PRO (CAS# 60207-90-1, PESTANAL® analytical standard, Sigma-Aldrich, purity: 99.2%) to prepare stock solutions respectively containing 3 mg FPF/g double-distilled H_2O, 1 mg DIM/g double-distilled H_2O, and 100 mg PRO/g acetone [5]. All bees, including control bees, were fed the same amount of solvent (0.7%). The solutions were maintained at 4 °C inside a bottle completely wrapped in aluminum foil to avoid light degradation. The stock solutions were diluted with 1.8 M sucrose solution (corresponding to 50% w/w) to prepare the final solutions that were fed to the bees.

Survival: synergistic and individual effects

Bee mortality was assessed 1 h, 2 h, 4 h, and each 24 h after treatment, up to a maximum of 72 h. A bee was considered dead when was immobile and did not react to any stimulation [4]. The LD_{50} of FPF, DIM, and FPF+PRO were estimated at 48 h after exposure [5]. Because of the very low effect of FPF alone at 375 ng FPF/bee, we only tested this FPF dose in combination with PRO.

Weight assessment

The effects of a given pesticide dose may depend upon bee weight. We therefore measured the weight of 354 pesticide-free bees. Because the amount of food ingested could influence the body weight, the bees were fed the same type of food (50% w/w sucrose solution, *ad libitum*) and frozen at the same time before weighing.

Statistical analysis

We used Fit Proportional Hazards models to separately test the effects of FPF, DIM, or FPF+PRO doses, season (early spring vs. summer), worker type (in-hive vs. forager bees), colony, and all interactions on bee survival (table S5). Because the FPF+PRO treatment was only tested in summer, we used the same model, but without season. Significant effects were further

analysed with Kaplan-Meier survival analyses (Wilcoxon Chi-square values) following visual data inspection.

Probit analysis [27] was used to estimate the LD_{50} of FPF, DIM, and FPF+PRO across season (early spring vs. summer) and worker type (in-hive vs. forager bees). Because the treatment FPF+PRO was only tested in summer, this treatment was not tested across seasons. Based on LD_{50} values, we defined biologically significant synergy as mixtures with minimum two-fold difference between observed and predicted effect concentrations using the Concentration Addition (CA) reference model [28]. We used the CA model because it is recommended for risk assessment purposes [29], and we used the two-fold difference limit to avoid false positives while focusing on synergistic effects of quantitative importance [28]. The ratio between predicted and observed effects of binary mixtures is defined as the Model Deviation Ratio (MDR) [30]. We used the MDR to define if the interaction of the chemical mixture FPF+PRO caused synergistic ($MDR > 2$), additive ($0.5 \leq MDR \leq 2$), or antagonistic ($MDR < 0.5$) effects [30]. The MDR was calculated using the Toxic Unit (TU) of the individual pesticides (FPF, PRO) and of the binary chemical mixture (FPF+PRO). The TU is defined as the ratio between the concentration of a mixture component and its toxicological acute (LD_{50}) endpoint [16]. Our TU calculations were based on the LD_{50} of FPF (our data, reported in the Results section; $LD_{50,\text{summer in-hive}} = 2995$ ng/bee, $LD_{50,\text{summer foragers}} = 1865$ ng/bee), PRO ($LD_{50} > 100000$ ng/bee [31]), and FPF+PRO (our data, reported in the Results section; $LD_{50,\text{summer in-hive}} = 758$ ng/bee, $LD_{50,\text{summer foragers}} = 353$ ng/bee). We calculated the TU of our chemical mixture (FPF+PRO) as the sum of the TUs of each individual chemical in the mixture.

We used a Mixed Model with a REML algorithm to test the effects of FPF or DIM doses, season (early spring vs. summer), worker type (in-hive vs. forager bees), and all interactions on the frequency of bees exhibiting at least one abnormal behaviour 1 h, 2 h, and 4 h after treatment (table S7-S8). Colony was included as a random factor. Because the FPF+PRO treatment was only tested in summer, we used the same model, but without season. We applied the square root-transformation on the frequency of bees exhibiting an abnormal behaviour. We determined the minimum dose that was significantly different from control using the Least-Square Means contrast test and visual data inspection.

We applied a binomial proportion model [32,33] to test for synergistic effects of FPF and PRO on bee survival (figure 1A-B) and behaviour (figure 3A-B). We used the additive effects

model [34], in which synergism is defined as the combined effect of multiple stressors significantly exceeding the sum of effects elicited by individual stressors. The R scripts (p.adjust function) used are available in the electronic supplementary material (ESM), and focused on testing synergistic, not antagonistic, effects. We used a script modified from Tosi *et al.* [33] and Sgolastra *et al.* [32] that tested for synergistic effects by testing if the difference between the observed and the expected effect (either mortality or presence of abnormal behaviour) of the combined treatment could arise by chance alone or was larger than the simple additive effect of both stressors.

The 0 ng/bee dose treatment was the control for each pesticide. Treatment A consisted of bees exposed only to each specific dose of FPF, for a total of four doses (750, 1500, 3000, 6000 ng/bee). Treatment B consisted of bees only exposed to PRO (7000 ng/bee). Bees exposed to both FPF and PRO (FPF+PRO) were assigned to the combined treatment (AB). We calculated the expected effect proportion of the combined treatment as $P_{ABExp} = P_A + (1-P_A) P_B$, where P_A and P_B are the observed effect proportions in the FPF and PRO treatments, respectively. We used Wald confidence intervals to build a hypothesis test for the difference between two proportions. We separately determined the synergistic effects at each assessment time and used the Holm method to correct for multiple comparisons ($\alpha = 0.05$), as recommended by the R script protocol (ESM). We tested the effects across two worker types (in-hive vs. foragers) during summer. We calculated the Risk Ratio (RR) and the Risk Difference (RD) to quantitatively express the size of the interactive effect of the chemical mixture on bee survival (frequency of dead bees, table S3) and behaviour (frequency of abnormally behaving bees, table S4) [35,36]. The RR was determined by dividing the observed effect by the expected effects (i.e. dividing the cumulative incidence in the exposed group by the cumulative incidence in the unexposed group) and therefore cannot be calculated when the expected effect is 0 [35,36]. To estimate the effect size of the pesticide mixture at all time points after treatment, we calculated the RD, the difference between the ratio of observed and expected effects (i.e. subtracting the cumulative incidence in the unexposed group from the cumulative incidence in the exposed group).

A Mixed Model (REML algorithm) was used to test the effects of season (early spring vs. summer), worker type (in-hive vs. forager bees), and all interactions on bee weight (table S10). Colony was included as a random factor.

Our statistical models were run with R v3.3.2 [37], JMP v10.0 (SAS Statistical Software), and Polo Plus v.2.0 (LeOra Software) software. We used residuals analysis to confirm that our data met parametric assumptions. We report mean ± 1 standard error (s.e.m.), and 95% Confidence Intervals for LD_{50} values [38]. We used an alpha value of 0.05. We applied the Dunn-Sidak method [39] to correct for multiple comparisons when appropriate, and indicated with ^{DS} the corrected statistical tests. We applied stepwise model simplification, building models with all interactions, and then removing them if they were not significant.

ESM Results

Our trials met the official guidelines for toxicity tests [5] because the 24 h LD_{50} of DIM was 114 ng/bee, within the required standard range of 100-350 ng/bee. Moreover, in-hive bees mortality of control treatments was low and within specified limits (≤10%) [5].

FPF doses up to 750 ng/bee caused little mortality (≤10%, not statistically different from control) when we applied the standard toxicological protocol (i.e. using in-hive bees), showing that FPF doses up to 750 ng/bee were sublethal [5].

DIM was more toxic in early spring

There was a significant effect of season on survival of bees exposed to DIM (Fit proportional hazards, $p < 0.0001$, tables S5-S6). DIM was significantly more toxic in early spring, as compared to summer. There were no significant interactions ($p > 0.13$). There was no significant seasonal effect of DIM LD_{50} (figure S2). There was a significant effect of the interaction dose × season or dose × season × worker type on bee abnormal behaviours after treatment with DIM (1-4 h: $p < 0.001$) (table S8).

There was a significant effect of season on bee abnormal behaviours 1 h and 2 h after treatment with DIM (Mixed $Model_{REML}$, $p < 0.0001$, table S8). There was no significant effect of season on bee behaviours at 4 h after treatment of DIM ($p > 0.62$).

Bee weight varied depending on worker type and season

There was a significant effect of worker type ($F_{1,351} = 44.66$, $p < 0.0001$) and season ($F_{1,350} = 5.58$, $p = 0.019$) on bee weight (table S10). In-hive bees were significantly heavier than

foragers (+11%), and summer bees were significantly heavier than early spring bees (+5%). There was no significant effect of the interaction worker type × season on pesticide-free bee weight ($p > 0.59$). The effect of colony accounted for 1% of model variance.

ESM Discussion

FPF and DIM showed opposite effects across season

Season consistently influenced pesticide toxicity (figures 2, 4, S2-S4), but its effect varied depending on the active ingredient. Bees were more susceptible to FPF in summer, while bees exposed to DIM were more affected in early spring (ESM). This variability is reflected in the results of prior studies, although we provide the first results showing how this variability can occur even when testing bees from the same apiary during the same seasons. Summer bees are typically more sensitive to pesticides as compared to winter bees [40–43]. However, the neonicotinoid imidacloprid reduced survival of winter bees, as compared to summer bees [41]. In early spring (first few weeks after overwintering), bees were more susceptible to the neonicotinoids, clothianidin and thiamethoxam, than summer bees [44]. The physiological modifications that bees experience across seasons, including variations in midgut structure (which can be a barrier to chemical transfer) and target receptor density, may account for some seasonal variability in pesticide toxicity [42,44]. Interestingly, the transition in bee sensitivity to pesticide seems to occur between early and late spring [44].

ESM tables

Table S1. Definitions of abnormal behaviours exhibited by bees. We provide a video showing examples of abnormal behaviours in the ESM, and additional details are in table S2.

Name	Definition
Motion coordination deficits	Loss of coordination consisting of falling or stumbling while walking, walking in circles, walking and flying with erratic and irregular movements, and bees that flap their wings while upside down.
Hyperactivity	Excitation manifested as rapid walking, sometimes including short jumps and flight attempts, fast movements of legs and antennae.
Apathy	Hypoactivity consisting of remaining largely motionless or walking very slowly. Such bees also have severely reduced or delayed reactions to stimulation provided by light, movements of other bees, or air currents (e.g. generated by nearby bees).
Curved-down abdomen	The abdomen is unnaturally curved and is flexed ventrally, cramps.
Moribund	The bee appears close to death and exhibits partial paralysis with slight movements of legs and antennae. Will respond slightly to mechanical stimulation.

Table S2. List of abnormal behaviours observed in the videos recorded during preliminary ecotoxicological trials to highlight and determine the types of common abnormal bee behaviours following pesticide consumption in sucrose solution. The video is available in the ESM (below) and Dryad Digital Repository, and further details are in table S1.

Video ID	Abnormal behaviour type	Description of abnormal behaviour(s)
1	Motion coordination deficits, hyperactivity	One bee shows hyperactivity, loss of coordination, stumbling, and erratic and irregular walking and flight movements.
2	Motion coordination deficits, hyperactivity	One bee is lying on the floor and shows loss of coordination with rapid twitching of legs and wings without flying for prolonged time.
3	Motion coordination deficits	One bee lies on the floor and shows rapid twitching of legs and wings without flying for prolonged time, loss of coordination.
4	Motion coordination deficits, hyperactivity	One bee shows hyperactivity, loss of coordination, stumbles, moves with erratic and irregular movements: atypical circular patterns.
5	Motion coordination deficits, hyperactivity	One bee lies on the floor and shows rapid twitching of legs and antennae, loss of coordination. The bees were recorded soon after exposure (acute oral) to the lower field-realistic dose of FPF (insecticide) and the sublethal dose of PRO (SBI fungicide; this fungicide dose alone caused no abnormal behaviours).
6	Hyperactivity	One bee (bottom, left) shows excitation and rapid movements of legs and antennae.
7	Apathy	Two hypoactive bees on the right side of the video.
8	Apathy	One hypoactive bee on the right side of the cage.
9	Curved-down abdomen, motion coordination deficits	One bee, standing next to the transparent cage door, showing a curved-down abdomen, and exhibiting irregular movements. Behind it, a bee rapidly twitches its legs and is unable to stand. On the left, moribund bees might seem dead but they exhibit partial paralysis and show slight movements of legs and antennae. This video shows a preliminary trial with bees exposed to the lower field-realistic dose of FPF and the sublethal dose of PRO (SBI fungicide) rapidly after acute oral treatment.
10	Moribund	One bee (close to cage front door) showing only slight movements of its legs and antennae, is unable to stand and appears close to death.

Table S3. Synergistic effects of FPF+PRO on bee survival, depending on FPF dose, worker type, and time after exposure (1-48 h). We show the Risk Ratio (RR, observed/expected), the Risk Difference (RD, observed-expected), and the statistical results (binomial proportion test, Holm corrected, $N = 420$). We report "NA" when the expected or observed mortality was 0. In this R script analysis, we only test for synergism.

Worker type	FPF dose (ng/bee)	PRO dose (ng/bee)	RR after treatment (h)					RD after treatment (h)					*P*-value after treatment (h)				
			1	2	4	24	48	1	2	4	24	48	1	2	4	24	48
In-hive	0	7000	NA	NA	NA	3.0	0.4	0	0	0	7	-15	1.000	1.000	1.000	0.741	1.000
	750		NA	8.0	9.0	5.5	2.0	23	23	27	44	29	**0.005**	**0.007**	**0.005**	**<0.001**	**0.013**
	1500		NA	4.5	3.3	3.3	2.0	23	23	30	37	29	**0.005**	**0.014**	**0.009**	**0.003**	**0.014**
	3000		4.5	3.0	0.9	1.3	1.3	23	20	-3	19	16	**0.036**	0.091	0.605	0.206	0.253
	6000		1.7	2.2	0.7	0.9	0.9	13	23	-20	-10	-7	0.475	0.112	1.000	1.000	1.000
Forager	0	7000	NA	NA	NA	0.2	0.3	0	0	-7	-27	-46	1.000	1.000	1.000	1.000	1.000
	750		NA	NA	NA	4.9	1.7	20	40	57	64	34	**0.006**	**<0.001**	**<0.001**	**<0.001**	**0.006**
	1500		5.0	1.4	1.5	1.4	1.2	27	10	17	19	13	**0.015**	0.387	0.351	0.351	0.387
	3000		11.0	2.6	1.4	1.3	1.1	33	27	20	22	11	**0.001**	**0.037**	0.107	0.107	0.202
	6000		1.0	1.2	0.8	1.0	1.0	0	7	-13	0	0	1.000	1.000	1.000	1.000	1.000

Table S4. Synergistic effects of FPF+PRO on the frequency of abnormal behaviours, depending on FPF dose, worker type, and time after exposure (1-4 h). We show the Risk Ratio (RR, observed/expected), the Risk Difference (RD, observed-expected), and the statistical results (binomial proportion test, Holm corrected, $N = 420$). We report "NA" when the expected or observed mortality was 0.

Worker type	FPF dose (ng/bee)	PRO dose (ng/bee)	RR after treatment (h)			RD after treatment (h)			*P*-value after treatment (h)		
			1	2	4	1	2	4	1	2	4
In-hive	0	7000	NA	NA	NA	0	0	0	1.000	1.000	1.000
	750		7.3	7.3	10.0	63	63	60	**<0.001**	**<0.001**	**<0.001**
	1500		1.7	2.3	2.3	23	33	27	**0.031**	**0.009**	**0.022**
	3000		0.8	1.0	1.6	-13	-3	23	1.000	1.000	0.094
	6000		1.0	0.7	2.0	-3	-27	27	1.000	1.000	**0.043**
Foragers	0	7000	NA	NA	NA	-7	-7	-7	1.000	1.000	1.000
	750		2.5	15.0	5.5	30	47	30	**0.005**	**<0.001**	**0.002**
	1500		0.7	1.9	2.3	-17	30	27	0.906	**0.022**	**0.022**
	3000		0.7	1.2	1.3	-20	10	7	0.954	0.654	0.654
	6000		1.8	1.1	0.7	30	7	-13	**0.021**	0.604	0.860

Table S5. Effect of dose, season, worker type, and all interactions on bee survival after exposure to FPF ($DF_{Model} = 20$), FPF+PRO ($DF_{Model} = 8$), or DIM ($DF_{Model} = 10$) (Fit proportional hazard). We report in bold the significant effects. For each factor, we report the statistical values of the last stepwise model simplification that included the factor.

Active ingredient	Factor	DF	L-R χ^2	*P*-value
FPF	Dose	5	297.74	**<0.0001**
	Season	1	29.11	**<0.0001**
	Worker type	1	49.60	**<0.0001**
	Dose × Season	5	14.98	**0.0105**
	Dose × Worker type	5	21.85	**0.0006**
	Season × Worker type	1	17.40	**<0.0001**
	Dose × Season × Worker type	5	4.90	0.4277
	Colony	2	5.86	0.0535
DIM	Dose	6	300.10	**<0.0001**
	Season	1	17.54	**<0.0001**
	Worker type	1	37.89	**<0.0001**
	Dose × Season	4	3.82	0.4306
	Dose × Worker type	6	5.76	0.4511
	Season × Worker type	1	2.25	0.1332
	Dose × Season × Worker type	5	2.69	0.6112
	Colony	2	0.22	0.8946
FPF+PRO	Dose	5	103.29	**<0.0001**
	Worker type	1	12.76	**0.0004**
	Dose × Worker type	5	1.32	0.9328
	Colony	2	3.15	0.2071

Table S6. Effect of dose, season, and worker type on bee survival, depending on FPF and DIM doses (Kaplan-Meier^{DS}, Wilcoxon). We report in bold the significant effects after Dunn-Sidak correction for multiple comparisons (FPF: dose and season, $k = 4$, adjusted $\alpha = 0.0127$; worker type: $k = 5$, adjusted $\alpha = 0.0102$; DIM: dose, $k = 6$, adjusted $\alpha = 0.0085$; season, $k = 4$, adjusted $\alpha = 0.0127$; worker type: $k = 5$, adjusted $\alpha = 0.0102$). For the dose effect, we compared all doses with control. For the seasonal effect, we did not use 25 and 800 ng DIM/bee in both seasons, since in summer we tested 25 ng DIM/bee instead of 800 ng DIM/bee because using the higher dose of DIM would have resulted in excessively high mortality precluding using this data ("NA"). We report the values of tested comparisons only.

		Dose effect			Season effect			Worker type effect		
Dose (ng/bee)		χ^2	DF	*P*-value	χ^2	DF	*P*-value	χ^2	DF	*P*-value
Control	0	NA						21.97	1	**<0.0001**
FPF	750									
	1500	3.89	1	**0.0487**	2.96	1	0.0855	14.29	1	**0.0002**
	3000	47.26	1	**<0.0001**	6.22	1	**0.0127**	18.48	1	**<0.0001**
	6000	115.97	1	**<0.0001**	13.23	1	**0.0003**	7.70	1	**0.0055**
	12000	155.89	1	**<0.0001**	0.08	1	0.7802	1.25	1	0.2629
DIM	25	8.11	1	**0.0044**	NA					
	50	10.17	1	**0.0014**	6.32	1	**0.012**	11.49	1	**0.0007**
	100	47.82	1	**<0.0001**	1.84	1	0.1744	17.47	1	**<0.0001**
	200	130.64	1	**<0.0001**	9.35	1	**0.0022**	9.38	1	**0.0022**
	400	153.32	1	**<0.0001**	16.99	1	**<0.0001**	15.76	1	**<0.0001**
	800	156.53	1	**<0.0001**	NA			4.80	1	**0.0285**

Table S7. Effect of FPF or FPF+PRO dose on the frequency of bees exhibiting at least one abnormal behaviour. Based on visual inspection of the data, we made limited tests of the effect of each pesticide dose as compared to control treatments (Mixed Model$_{REML}$, Contrast test^{DS}). We report only tested comparisons.

Active ingredient (name)	Time from treatment (h)	FPF dose (ng/bee)	DF numerator	DF denominator	*F* Ratio	*P*-value
FPF	1	750	1	52	6.96	**0.0109**
		1500	1	52	106.60	**<0.0001**
		3000	1	52	182.58	**<0.0001**
		6000	1	52	193.95	**<0.0001**
		12000	1	52	232.27	**<0.0001**
	2	1500	1	62	24.41	**<0.0001**
		3000	1	62	42.44	**<0.0001**
		6000	1	62	56.77	**<0.0001**
		12000	1	62	67.75	**<0.0001**
	4	1500	1	60	16.07	**0.0002**
		3000	1	60	32.51	**<0.0001**
		6000	1	60	46.74	**<0.0001**
		12000	1	60	18.98	**<0.0001**
FPF+PRO	1	375	1	32	164.81	**<0.0001**
		750	1	32	176.36	**<0.0001**
		1500	1	32	145.40	**<0.0001**
		3000	1	32	176.68	**<0.0001**
		6000	1	32	194.62	**<0.0001**
	2	375	1	32	228.32	**<0.0001**
		750	1	32	276.09	**<0.0001**
		1500	1	32	279.59	**<0.0001**
		3000	1	32	279.07	**<0.0001**
		6000	1	32	241.88	**<0.0001**
	4	375	1	25	148.24	**<0.0001**
		750	1	25	166.23	**<0.0001**
		1500	1	25	157.22	**<0.0001**
		3000	1	25	146.20	**<0.0001**
		6000	1	25	133.70	**<0.0001**

Table S8. Abnormal behaviours of bees exposed to FPF, FPF+PRO, and DIM, depending on season, worker type, doses, and time after exposure. The effect of FPF+PRO was not tested across season. We tested the effects up to 4 h after treatment because the high mortality elicited by the pesticides at 24-48 h limited the sample size and precluded a rigorous analysis of abnormal behaviours.

Active ingredient	Time after treatment (h)	Colony effect (%)	Factor	DF numerator	DF denominator	*F* Ratio	*P*-value
FPF	1	5	Dose	5	52	82.54	**<0.0001**
			Season	1	52	25.74	**<0.0001**
			Worker type	1	52	4.16	**0.0464**
			Dose × Season	5	52	5.43	**0.0004**
			Dose × Worker type	5	52	4.90	**0.0010**
			Season × Worker type	1	46	1.61	0.2115
			Dose × Season × Worker type	5	46	2.25	0.0654
	2	<1	Dose	5	62	25.60	**<0.0001**
			Season	1	62	7.74	**0.0071**
			Worker type	1	62	0.13	0.7155
			Dose × Season	5	46	0.25	0.9354
			Dose × Worker type	5	46	2.44	**0.0486**
			Season × Worker type	1	46	2.75	0.1041
			Dose × Season × Worker type	5	46	2.40	0.0515
	4	1	Dose	5	60	14.99	**<0.0001**
			Season	1	60	0.43	0.5161
			Worker type	1	60	0.31	0.5798
			Dose × Season	5	44	0.71	0.6221
			Dose × Worker type	5	44	1.15	0.3499
			Season × Worker type	1	44	0.08	0.7759
			Dose × Season × Worker type	5	44	1.48	0.2144

Active ingredient	Time after treatment (h)	Colony effect (%)	Factor	DF numerator	DF denominator	*F* Ratio	*P*-value
DIM	1	<1	Dose	5	47	19.68	**<0.0001**
			Season	1	47	31.71	**<0.0001**
			Worker type	1	47	27.97	**<0.0001**
			Dose × Season	5	47	5.24	**0.0007**
			Dose × Worker type	5	47	2.84	**0.0254**
			Season × Worker type	1	46	2.28	0.1382
			Dose × Season × Worker type	5	47	2.97	**0.0208**
	2	<1	Dose	5	52	81.00	**<0.0001**
			Season	1	52	44.43	**<0.0001**
			Worker type	1	52	8.65	**0.0049**
			Dose × Season	5	46	1.15	0.3501
			Dose × Worker type	5	52	5.43	**0.0004**
			Season × Worker type	1	46	0.36	0.5515
			Dose × Season × Worker type	5	52	5.23	**0.0006**
	4	2	Dose	5	44	45.40	**<0.0001**
			Season	1	44	0.24	0.6241
			Worker type	1	44	0.08	0.7775
			Dose × Season	5	44	5.06	**0.0009**
			Dose × Worker type	5	44	8.90	**0.0000**
			Season × Worker type	1	44	4.87	**0.0325**
			Dose × Season × Worker type	5	44	5.05	**0.0010**
FPF+PRO	1	<1	Dose	6	32	82.17	**<0.0001**
			Worker type	1	32	1.09	0.3052
			Dose × Worker type	6	26	0.68	0.6662
	2	<1	Dose	6	32	124.83	**<0.0001**
			Worker type	1	32	0.24	0.6289
			Dose × Worker type	6	26	1.35	0.2714
	4	28	Dose	6	25	72.59	**<0.0001**
			Worker type	1	25	4.00	0.0565
			Dose × Worker type	6	25	4.56	**0.0030**

Table S9. The effects of interactions dose × worker type and dose × season on the abnormal behaviours of bees depending on FPF or FPF+PRO exposure. Based upon visual inspection of the data, we conducted limited tests of the effect of worker type (in-hive vs. forager bees) or season (early spring vs. summer) at each specific dose treatment (Mixed Model$_{REML}$, Contrast test^{DS}). The effect of FPF+PRO was not tested across season. We report only tested comparisons.

Effect	Active ingredient (name)	Time from treatment (h)	FPF dose (ng/bee)	DF numerator	DF denominator	*F* Ratio	*P*-value
Dose × Worker type	FPF	1	1500	1	52	17.16	**0.0001**
	FPF	1	3000	1	52	3.35	0.0729
	FPF+PRO	4	375	1	25	9.09	**0.0058**
Dose × Season	FPF	1	750	1	52	26.86	**<0.0001**
	FPF	1	1500	1	52	6.73	**0.0123**
	FPF	1	3000	1	52	13.96	**0.0005**

Table S10. Effect of season, worker type, and their interaction on bee weight. For each factor, we report the statistical values of the latest possible stepwise model simplification (Mixed $Model_{REML}$).

Factor	N	R^2	Colony effect (%)	DF numerator	DF denominator	*F* Ratio	*P*-value
Season				1	350	5.58	**0.0187**
Worker type	354	0.15	1	1	351	44.66	**<0.0001**
Season × Worker type				1	350	0.28	0.5974

ESM figures

Figure S1. The LD_{50} (48 h) of bees exposed to FPF (left and centre) and FPF+PRO (right) across seasons (early spring vs. summer) and worker types (in-hive bees vs. foragers). Above each bar, we show the LD_{50} values. Different letters indicate significant differences. We show the 24 h LD_{50} of summer foragers (light grey bars), because high summer forager mortality at 48 h prevented the accurate estimation of their 48 h LD_{50} (standard LD_{50} estimation time, dark grey bars). Error bars represent 95% confidence intervals ($N_{overall}$ = 1080).

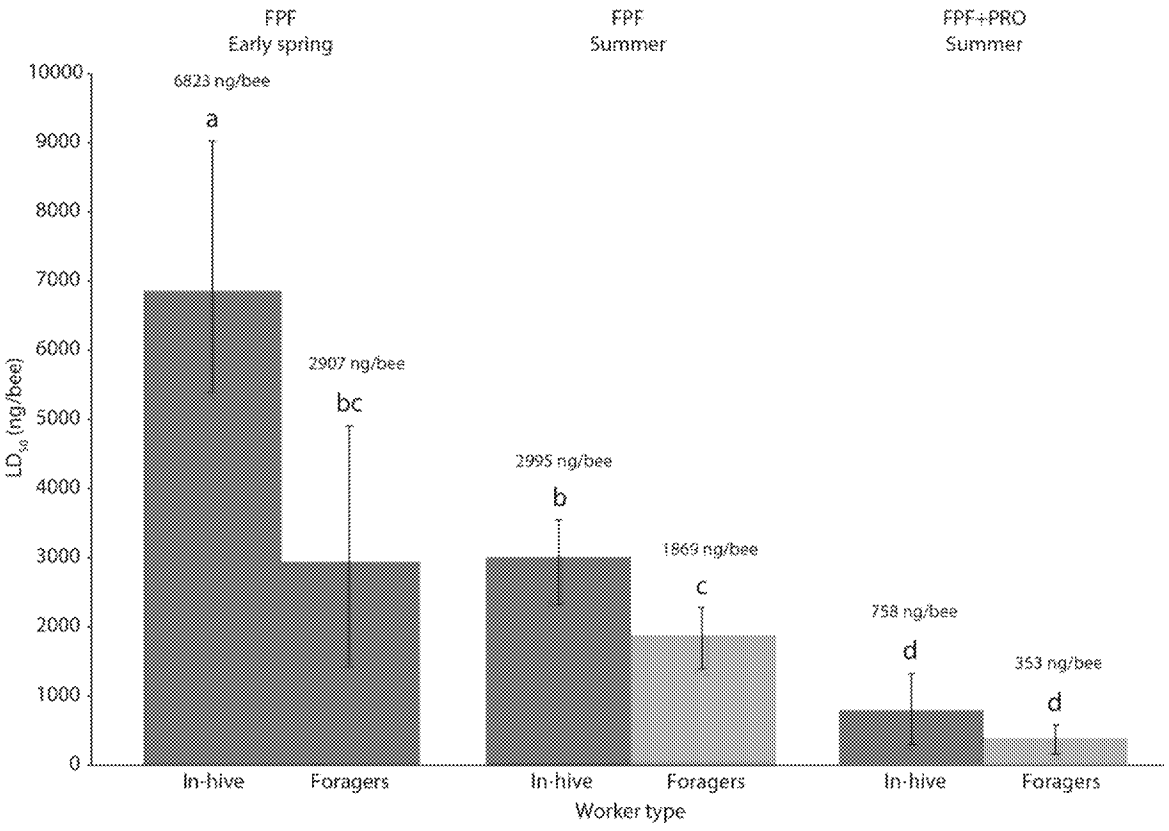


Figure S2. The LD_{50} (48 h) of bees exposed to DIM across seasons (early spring vs. summer) and worker types (in-hive bees vs. foragers). Above each bar, we show the LD_{50} values. Different letters indicate significant differences. We show the 24 h LD_{50} of summer foragers (light gray bars), because high forager mortality of summer foragers at 48 h prevented the accurate estimation of their 48 h LD_{50}. Error bars represent 95% confidence intervals.

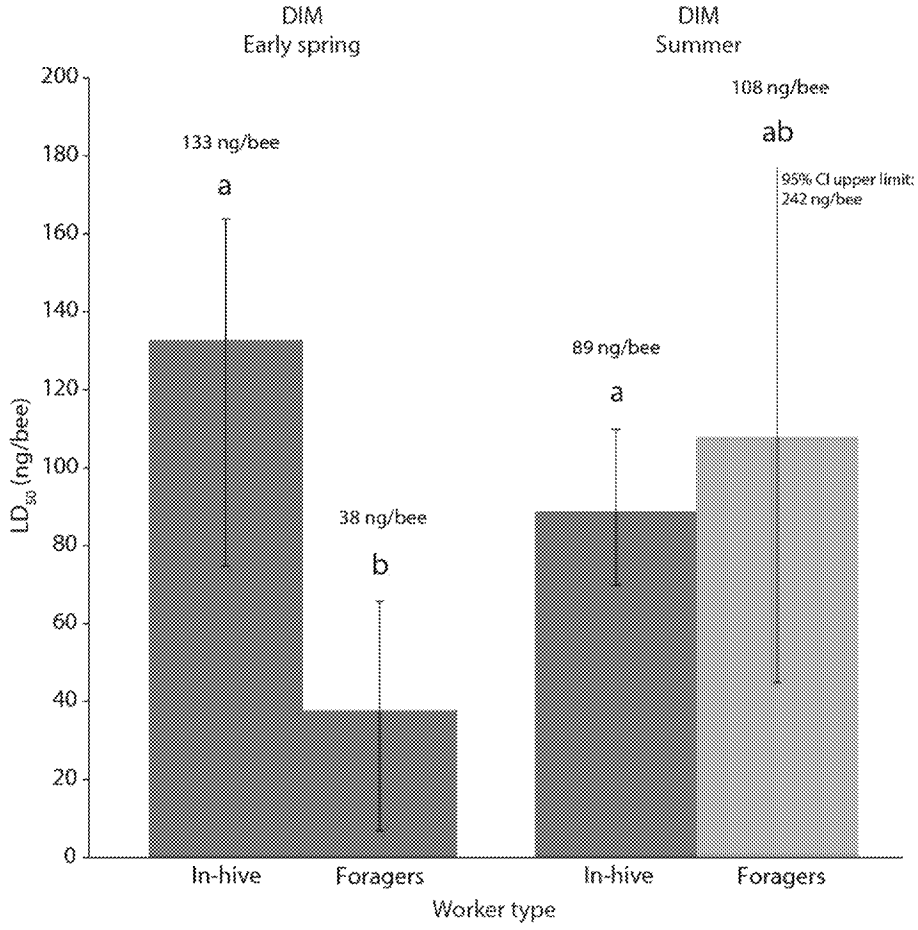


Figure S3. Main effects of (A, D) dose, (B, E) season, and (C, F) worker type on survival of bees exposed to (A, B, C) FPF or (D, E, F) DIM. Asterisks indicate significant differences (Kaplan-Meier^{DS}, *$p < 0.05$, **$p < 0.01$, **** $p < 0.0001$, table S6). In A and D, we made limited pairwise comparisons testing the dose effect (0-5 dose levels, corresponding to a control dose and between 750-12000 ng FPF/bee in A, 50-800 ng DIM/bee in D) comparing each dose to control based upon visual inspection of the data (Dunn-Sidak corrected, table S6).

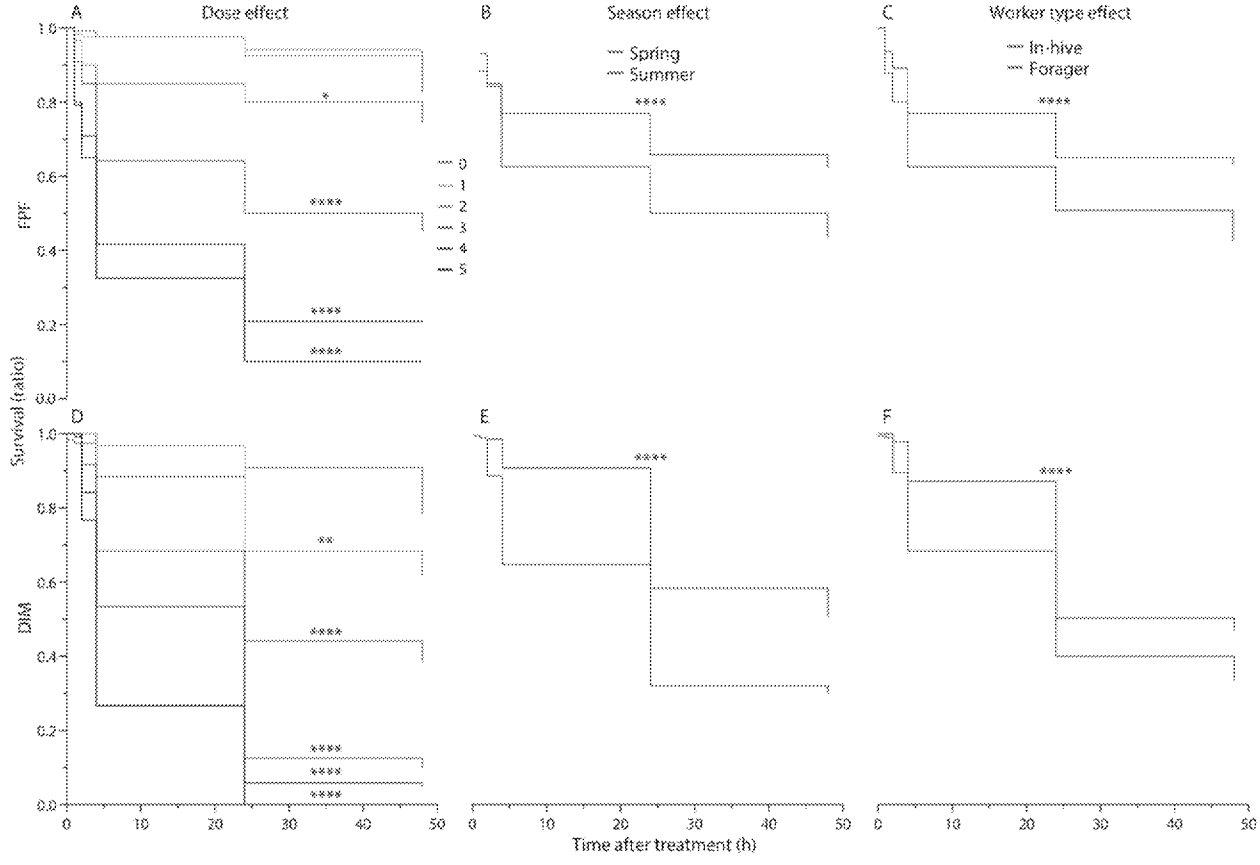
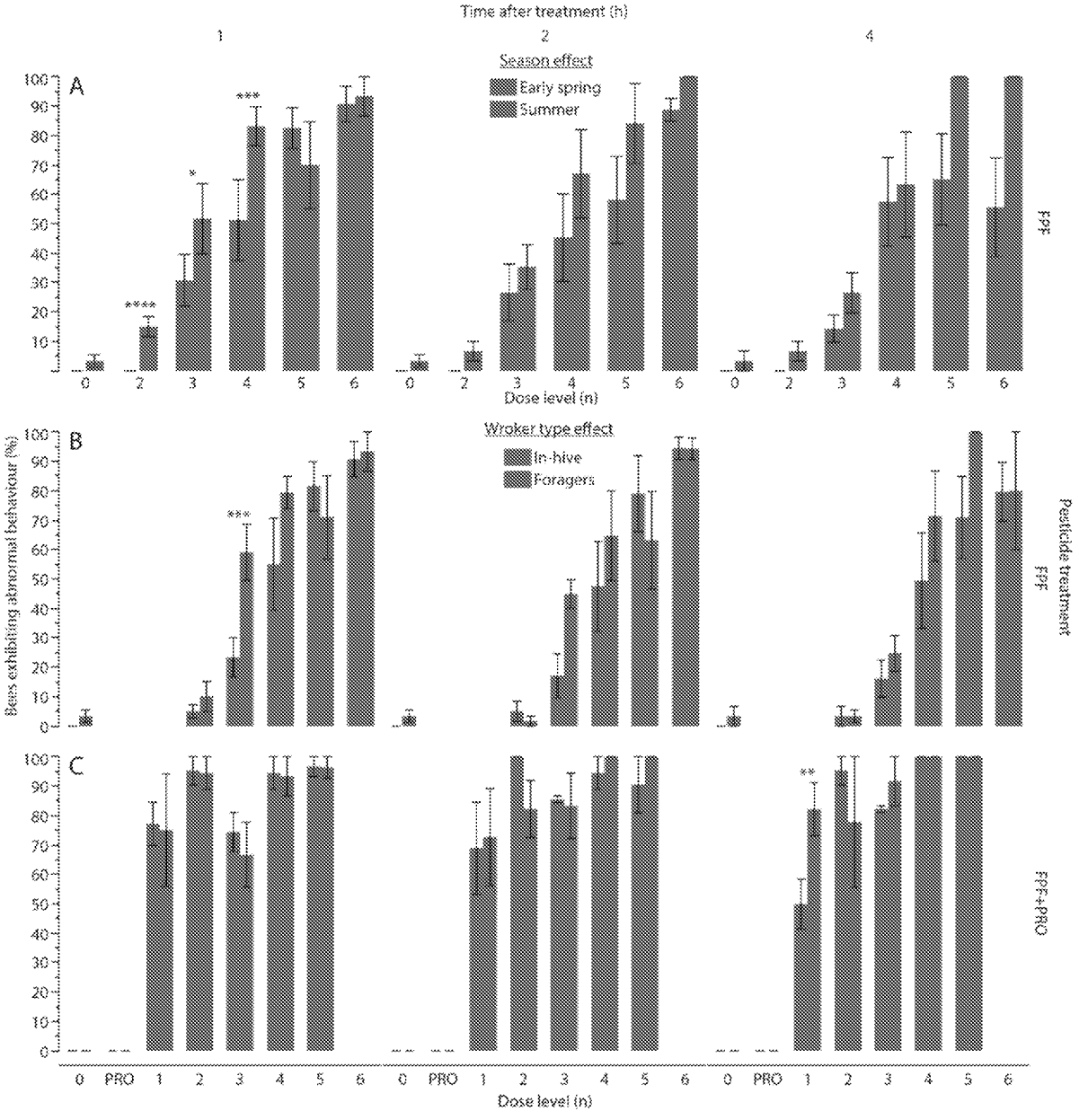


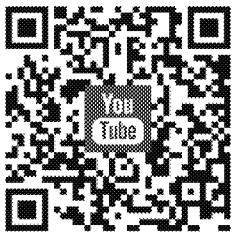
Figure S4. Effect of (A) season and (B, C) worker type on the frequency of bees exhibiting abnormal behaviours after exposure to (A, B) FPF or (C) FPF+PRO doses (0-6 dose levels, corresponding to a control dose and between 375-12000 ng FPF/bee). In figure 3C-D of the main text, these results were pooled by worker type, but are here split by worker type to provide further information. Asterisks indicate significant differences (Mixed Model$_{REML}$, Contrast test^{DS}, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). We compared the effect of season and worker type within each pesticide dose based on visual estimation (Dunn-Sidak corrected). Main effects and further statistical details are reported in tables S8-S9.



ESM videos

Abnormal behaviours observed in videos recorded during preliminary ecotoxicological trials. The videos highlight and better define the types of common abnormal bee behaviours occurring after oral pesticide consumption in sucrose solution. Further details are available in table S1-S2. The video is available in:

- ✓ the Dryad Digital Repository (https://doi.org/10.5061/dryad.5f87k5v)
- ✓ at this YouTube link: YouTube Video of Abnormal Behaviours
- ✓ at this QR code:



R scripts

R script for testing synergistic effects on survival

```
##############################################################################
# Testing the synergistic effects of two chemicals on survival
# Testing for additivity:
#    Confidence interval for binomial proportion difference under Bliss independence.
#
# INPUTS:
# ndead = vector with 3 elements, containing number of dead individuals under
#         treatment A, B and combined.
# ntot  = vector with 3 elements, containing total number of individuals under
#         the 3 treatments.
# p.signif = significance level (usually 0.05).
# alternative = character string specifying the alternative hypothesis.
#
# OUTPUTS:
# See Tosi et al. 2019
##############################################################################

ci.bliss.additivity <- function(ndead,ntot,p.signif=0.05,alternative="greater") {
 if (alternative=="two.sided") p.signif <- p.signif/2  # Two-tailed test.
 ndead <- unname(ndead)
 ntot <- unname(ntot)
 p <- ndead/ntot
 pa <- p[1]
 pb <- p[2]
 pab.obs <- p[3]
 vara <- p[1]*(1-p[1])/ntot[1]
 varb <- p[2]*(1-p[2])/ntot[2]
 varab.obs <- p[3]*(1-p[3])/ntot[3]
```

```
  pab.exp <- pa+pb-pa*pb
  varab.exp <- vara+varb+pb^2*vara+pa^2*varb    # Derived with the Delta method.
  p.dif <- pab.obs-pab.exp
  sd.all <- sqrt(varab.obs+varab.exp)
  z <- qnorm(1-p.signif)
  out <- list(pA=pa,pB=pb,pAB.obs=pab.obs,pAB.exp=pab.exp,p.Dif=p.dif,
          VarA=vara,VarB=varb,VarAB.obs=varab.obs,VarAB.exp=varab.exp,Var.All=sd.all^2,
          CI=switch(alternative,
                  two.sided=c(lower=p.dif-z*sd.all,upper=p.dif+z*sd.all),
                  less=c(upper=p.dif+z*sd.all),
                  greater=c(lower=p.dif-z*sd.all)))
  return(out)
}

# Calculates the exact p-value by inverting the hypothesis test.
invert.hypothesis.bliss <- function(n.mort,n.total) {
  fbliss <- function(signif)
ci.bliss.additivity(n.mort,n.total,signif,alternative="greater")$CI["lower"]
  loglik <- function(signif) abs(fbliss(signif))
  return(optimize(loglik,interval=c(0,1),maximum=F,tol=1e-32)$minimum)
}

# Mortality data. Column 1 (e.g. datamort[[1]][,1]) contains the total number of individuals,
labelled "N".
datamort <- list()
datamort[[1]] <- cbind(c(30,30,30),c(0,0,0),c(0,0,0),c(2,0,0),c(7,4,2),c(13,10,5)) # forager, 0
datamort[[2]] <- cbind(c(30,30,30),c(0,0,6),c(0,0,12),c(0,0,17),c(1,4,24),c(7,10,25)) # forager,
750
datamort[[3]] <- cbind(c(30,30,30),c(2,0,10),c(7,0,10),c(10,0,15),c(13,4,21),c(15,10,24)) #
forager, 1500
```

```
datamort[[4]] <- cbind(c(30,30,30),c(1,0,11),c(5,0,13),c(16,0,22),c(20,4,28),c(25,10,30)) #
forager, 3000
datamort[[5]] <- cbind(c(30,30,30),c(8,0,8),c(11,0,13),c(25,0,21),c(30,4,30),c(30,10,30)) #
forager, 6000
datamort[[6]] <- cbind(c(30,30,30),c(0,0,0),c(0,0,0),c(0,0,0),c(0,1,3),c(3,5,3)) # in-hive, 0
datamort[[7]] <- cbind(c(30,30,30),c(0,0,7),c(1,0,8),c(1,0,9),c(2,1,16),c(4,5,17)) # in-hive, 750
datamort[[8]] <- cbind(c(30,30,30),c(0,0,7),c(2,0,9),c(4,0,13),c(4,1,16),c(4,5,17)) # in-hive, 1500
datamort[[9]] <- cbind(c(30,30,30),c(2,0,9),c(3,0,9),c(12,0,11),c(17,1,23),c(17,5,24)) # in-hive,
3000
datamort[[10]] <- cbind(c(30,30,30),c(6,0,10),c(6,0,13),c(22,0,16),c(29,1,26),c(29,5,27)) # in-
hive, 6000

for (i in 1:10) rownames(datamort[[i]]) <- c("TREAT.A","TREAT.B","TREAT.AB") #
TREAT.A = FPF; TREAT.B = PRO; TREAT.AB = FPF+PRO
for (i in 1:10) colnames(datamort[[i]]) <- c("N","1h","2h","4h","24h","48h")

cat("------------------------------------------------------------\n")

# Testing Bliss additivity. All we need to do is to define "n.total" and "n.mort", and then feed
invert.hypothesis.bliss() with those two numbers.
# Index i runs from 1 to the number of synergies tested (=1).
# For a generic dataset with 1 endpoint and where nt=total number of individuals and nd=number
of dead individuals, we would do: p <- invert.hypothesis.bliss(nt,nd)

for (i in 1:10) {
  a <- datamort[[i]]
  b <- a[,-1]
  p.value <- NULL

# For each endpoint j we test the Bliss hypothesis.
  for (j in 1:5) {
```

```
   n.total <- a[c(1,2,3),1]    # Total number of individuals
   n.mort <- a[c(1,2,3),j+1]   # Number of dead individuals.
   p <- invert.hypothesis.bliss(n.mort,n.total)  # p-value from inverting the hypothesis test.
   p.value <- c(p.value,p)
  }
# Control for multiple comparison, Holm methodology. For cases where there is only 1 endpoint this is obviously not needed.
  p.correct <- p.adjust(p.value,method="holm")

# Formatted output.
  name.data <- c("forager, 0","forager, 750","forager, 1500","forager, 3000","forager, 6000","in-hive, 0","in-hive, 750","in-hive, 1500","in-hive, 3000","in-hive, 6000")
  cat(paste(name.data[i],"\n",sep=""))
  names(p.correct) <- c("1h","2h","4h","24h","48h")
  print(datamort[[i]])
  cat("\n")
  cat(paste(name.data[i],". Observed and expected binomial proportions.\n",sep=""))
  pab <- a[,-1]/a[,1]
  pab <- rbind(pab,pab[1,]+pab[2,]-pab[1,]*pab[2,])
  rownames(pab) <- c("TREAT.A","TREAT.B","TREAT.AB","Expected")
  print(pab)
  cat("\n")
  cat(paste(name.data[i],". Control of type I errors (Holm method) in binomial proportion test.\n",sep=""))
  print(p.correct)

  cat("------------------------------------------------------------\n")
}
```

R script for testing synergistic effects on abnormal behaviours

```
##############################################################################
```

```
# Testing the synergistic effects of two chemicals on abnormal behaviours
# Testing for additivity:
#    Confidence interval for binomial proportion difference under Bliss independence.
#
# INPUTS:
# nabnbe = vector with 3 elements, containing number of individuals exhibiting abnormal
#        behaviour under treatment A, B and combined.
# ntot  = vector with 3 elements, containing total number of individuals under
#        the 3 treatments.
# p.signif = significance level (usually 0.05).
# alternative = character string specifying the alternative hypothesis.
#
# OUTPUTS:
# See Tosi et al. 2019
##############################################################################

ci.bliss.additivity <- function(nabnbe,ntot,p.signif=0.05,alternative="greater") {
 if (alternative=="two.sided") p.signif <- p.signif/2  # Two-tailed test.
 nabnbe <- unname(nabnbe)
 ntot <- unname(ntot)
 p <- nabnbe/ntot
 pa <- p[1]
 pb <- p[2]
 pab.obs <- p[3]
 vara <- p[1]*(1-p[1])/ntot[1]
 varb <- p[2]*(1-p[2])/ntot[2]
 varab.obs <- p[3]*(1-p[3])/ntot[3]
 pab.exp <- pa+pb-pa*pb
 varab.exp <- vara+varb+pb^2*vara+pa^2*varb    # Derived with the Delta method.
 p.dif <- pab.obs-pab.exp
 sd.all <- sqrt(varab.obs+varab.exp)
```

```
  z <- qnorm(1-p.signif)
  out <- list(pA=pa,pB=pb,pAB.obs=pab.obs,pAB.exp=pab.exp,p.Dif=p.dif,
          VarA=vara,VarB=varb,VarAB.obs=varab.obs,VarAB.exp=varab.exp,Var.All=sd.all^2,
          CI=switch(alternative,
                two.sided=c(lower=p.dif-z*sd.all,upper=p.dif+z*sd.all),
                less=c(upper=p.dif+z*sd.all),
                greater=c(lower=p.dif-z*sd.all)))
  return(out)
}

# Calculates the exact p-value by inverting the hypothesis test.
invert.hypothesis.bliss <- function(n.abnbe,n.total) {
  fbliss <- function(signif)
ci.bliss.additivity(n.abnbe,n.total,signif,alternative="greater")$CI["lower"]
  loglik <- function(signif) abs(fbliss(signif))
  return(optimize(loglik,interval=c(0,1),maximum=F,tol=1e-32)$minimum)
}

# Abnormal behaviour data (individuals exhibiting the behaviour).
# Column 1 (e.g. dataabnbe[[1]][,1]) contains the total number of individuals, labelled "N".
dataabnbe <- list()

dataabnbe[[1]] <- cbind(c(30,30,30),c(0,0,0),c(0,0,0),c(0,0,0)) # in-hive, 0
dataabnbe[[2]] <- cbind(c(30,30,30),c(3,0,22),c(3,0,22),c(2,0,20)) # in-hive, 750
dataabnbe[[3]] <- cbind(c(30,30,30),c(10,0,17),c(8,0,18),c(6,0,14)) # in-hive, 1500
dataabnbe[[4]] <- cbind(c(30,30,30),c(24,0,20),c(21,0,20),c(12,0,19)) # in-hive, 3000
dataabnbe[[5]] <- cbind(c(30,30,30),c(21,0,20),c(23,0,15),c(8,0,16)) # in-hive, 6000
dataabnbe[[6]] <- cbind(c(30,30,30),c(2,0,0),c(2,0,0),c(2,0,0)) # foragers, 0
dataabnbe[[7]] <- cbind(c(30,30,30),c(6,0,15),c(1,0,15),c(2,0,11)) # foragers, 750
dataabnbe[[8]] <- cbind(c(30,30,30),c(19,0,14),c(10,0,19),c(6,0,14)) # foragers, 1500
dataabnbe[[9]] <- cbind(c(30,30,30),c(23,0,17),c(14,0,17),c(6,0,8)) # foragers, 3000
```

```
dataabnbe[[10]] <- cbind(c(30,30,30),c(12,0,21),c(15,0,17),c(13,0,9)) # foragers, 6000

for (i in 1:10) rownames(dataabnbe[[i]]) <- c("TREAT.A","TREAT.B","TREAT.AB") #
TREAT.A = FPF; TREAT.B = PRO; TREAT.AB = FPF+PRO
for (i in 1:10) colnames(dataabnbe[[i]]) <- c("N","1h","2h","4h")

cat("----------------------------------------------------------------\n")

# Testing Bliss additivity. All we need to do is to define "n.total" and "n.abnbe",
#                          and then feed invert.hypothesis.bliss() with those two numbers.
# Index i runs from 1 to the number of synergies tested.
# For a generic dataset with 1 endpoint and where nt=total number of individuals
#                          and nab=number of individuals exhibiting abnormal behaviour,
#                                  we would do: p <- invert.hypothesis.bliss(nt,nab)

for (i in 1:10) {
 a <- dataabnbe[[i]]
 b <- a[,-1]
 p.value <- NULL

# For each endpoint j we test the Bliss hypothesis. J is the # of time assessments
 for (j in 1:3) {
  n.total <- a[c(1,2,3),1]   # Total number of individuals
  n.abnbe <- a[c(1,2,3),j+1]  # Number of bees exhibiting abnormal behaviour.
  p <- invert.hypothesis.bliss(n.abnbe,n.total)  # p-value from inverting the hypothesis test.
  p.value <- c(p.value,p)
 }
# Control for multiple comparison, Holm methodology. For cases where there is only 1 endpoint
this is obviously not needed.
 p.correct <- p.adjust(p.value,method="holm")
```

```
# Formatted output.
  name.data <- c("in-hive, 0","in-hive, 750","in-hive, 1500","in-hive, 3000","in-hive,
6000","foragers, 0","foragers, 750","foragers, 1500","foragers, 3000","foragers, 6000")
  cat(paste(name.data[i],"\n",sep=""))
  names(p.correct) <- c("1h","2h","4h")
  print(dataabnbe[[i]])
  cat("\n")
  cat(paste(name.data[i],". Observed and expected binomial proportions.\n",sep=""))
  pab <- a[,-1]/a[,1]
  pab <- rbind(pab,pab[1,]+pab[2,]-pab[1,]*pab[2,])
  rownames(pab) <- c("TREAT.A","TREAT.B","TREAT.AB","Expected")
  print(pab)
  cat("\n")
  cat(paste(name.data[i],". Control of type I errors (Holm method) in binomial proportion
test.\n",sep=""))
  print(p.correct)

  cat("------------------------------------------------------------\n")
}
```

ESM references

1. Dietemann V *et al.* 2013 Standard methods for varroa research. *J. Apic. Res.* **52**, 1–54. (doi:10.3896/IBRA.1.52.1.09)
2. Fries I *et al.* 2013 Standard methods for nosema research. *J. Apic. Res.* **52**, 1–28. (doi:10.3896/IBRA.1.52.1.14)
3. Delaplane KS, Steen J Van Der, Guzman-novoa E. 2013 Standard methods for estimating strength parameters of *Apis mellifera* colonies. *J. Apic. Res.* **52**, 1–12. (doi:10.3896/IBRA.1.52.1.03)
4. Medrzycki P *et al.* 2013 Standard methods for toxicology research in *Apis mellifera*. *J. Apic. Res.* **52**, 1–60. (doi:10.3896/IBRA.1.52.4.14)

5. OECD/OCDE. 1998 OECD Guideline 213 for the testing of chemicals: honeybees, acute oral toxicity test. (doi:10.1787/9789264070165-en)
6. Williams GR *et al.* 2013 Standard methods for maintaining adult *Apis mellifera* in cages under in vitro laboratory conditions. *J. Apic. Res.* **52**, 1–36. (doi:10.3896/IBRA.1.52.1.04)
7. Jeschke P, Nauen R, Gutbrod O, Beck ME, Matthiesen S, Haas M, Velten R. 2015 Flupyradifurone (Sivanto^{TM}) and its novel butenolide pharmacophore: Structural considerations. *Pestic. Biochem. Physiol.* **121**, 31–38. (doi:10.1016/j.pestbp.2014.10.011)
8. Nauen R *et al.* 2014 Flupyradifurone: a brief profile of a new butenolide insecticide. *Pest Manag. Sci.* **71**, n/a-n/a. (doi:10.1002/ps.3932)
9. US EPA. 2015 Notice of pesticide registration for flupyradifurone, EPA reg. no. 264-1143. Office of Pesticide Programs, Environmental Fate and Effects Division EFED, Environmental Risk Branch IV 1–6.
10. EFSA. 2016 Setting of new maximum residue levels for flupyradifurone in strawberries, blackberries and raspberries. *EFSA J.* **14**. (doi:10.2903/j.efsa.2016.4423)
11. Bayer CropScience AG. 2018 Sivanto worldwide. *www.sivanto.bayer.com/sivanto-worldwide.html*. See https://www.sivanto.bayer.com/sivanto-worldwide.html (accessed on 23 March 2018).
12. Campbell JW, Cabrera AR, Stanley-Stahr C, Ellis JD. 2016 An evaluation of the honey bee (Hymenoptera: Apidae) safety profile of a new systemic insecticide, flupyradifurone, under field conditions in Florida. *J. Econ. Entomol.* **96**, 875–878. (doi:10.1093/jee/tow186)
13. US EPA. 2014 Environmental fate and ecological risk assessment for foliar, soil drench, and seed treatment uses of the new insecticide flupyradifurone (BYI 02960).
14. Bayer CropScience AG. 2013 Flupyradifurone technical information.
15. US EPA. 2012 White paper in support of the proposed risk assessment process for bees.
16. EFSA. 2013 EFSA Guidance Document on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). *EFSA J.* **11**, 268. (doi:10.2903/j.efsa.2013.3295)
17. Crane E. 1975 *Honey. A comprehensive survey*. London: Heinemann.
18. Pierre J, Mesquida J, Marilleau R, Pham-Delègue MH, Renard M. 1999 Nectar secretion in winter oilseed rape, Brassica napus - Quantitative and qualitative variability among 71

genotypes. *Plant Breed.* **118**, 471–476. (doi:10.1046/j.1439-0523.1999.00421.x)

19. Knopper LD, Dan T, Reisig DD, Johnson JD, Bowers LM. 2016 Sugar Concentration in Nectar: A Quantitative Metric of Crop Attractiveness for Refined Pollinator Risk Assessments. *Pest Manag. Sci.* (doi:10.1002/ps.4321)
20. Syngenta. 2009 QuiltXcel - Broad spectrum fungicide for control of plant diseases.
21. US EPA. 2017 Propiconazole EC.
22. Knezevic S. 2017 *2017 Guide for weed, disease, and insect management in Nebraska*. The Board of Regents of the University of Nebraska–Lincoln.
23. Tosi S, Costa C, Vesco U, Quaglia G, Guido G. 2018 A 3-year survey of Italian honey bee-collected pollen reveals widespread contamination by agricultural pesticides. *Sci. Total Environ.* **615**, 208–218. (doi:10.1016/j.scitotenv.2017.09.226)
24. Milner AM, Boyd IL. 2017 Toward pesticidovigilance. *Science (80-.).* **357**, 1232–1234. (doi:10.1126/science.aan2683)
25. EFSA. 2012 Scientific Opinion on the science behind the development of a risk assessment of Plant Protection Products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). *EFSA J.* **10**, 1–275. (doi:10.2903/j.efsa.2012.2668)
26. EFSA. 2014 Towards an integrated environmental risk assessment of multiple stressors on bees : review of research projects in Europe, knowledge gaps and. **12**, 1–102. (doi:10.2903/j.efsa.2014.3594)
27. Russell RM, Robertson JL, Savin NE. 1977 POLO: A new computer program for Probit analysis. *Bull. Entomol. Soc. Am.* **23**, 209–213. (doi:10.1093/besa/23.3.209)
28. Cedergreen N. 2014 Quantifying synergy: A systematic review of mixture toxicity studies within environmental toxicology. *PLoS One* **9**. (doi:10.1371/journal.pone.0096580)
29. Backhaus T, Faust M. 2012 Predictive environmental risk assessment of chemical mixtures: a conceptual framework. *Environ. Sci. Technol.* (doi:10.1021/es2034125)
30. Belden JB, Gilliom RJ, Lydy MJ. 2007 How well can we predict the toxicity of pesticide mixtures to aquatic life? *Integr. Environ. Assess. Manag.* **3**, 364–372. (doi:10.1897/1551-3793(2007)3[364:HWCWPT]2.0.CO;2)
31. Lewis KA, Tzilivakis J, Warner DJ, Green A. 2016 An international database for pesticide risk assessments and management. *Hum. Ecol. Risk Assess.* **22**, 1050–1064. (doi:10.1080/10807039.2015.1133242)

32. Sgolastra F *et al.* 2017 Synergistic mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting fungicide in three bee species. *Pest Manag. Sci.* **73**, 1236–1243. (doi:10.1002/ps.4449)
33. Tosi S, Nieh JC, Sgolastra F, Cabbri R, Medrzycki P. 2017 Neonicotinoid pesticides and nutritional stress synergistically reduce survival in honey bees. *Proc. R. Soc. B Biol. Sci.* **284**, 20171711. (doi:10.1098/rspb.2017.1711)
34. Folt C, Chen C. 1999 Synergism and antagonism among multiple stressors. *Limnol. Oceanogr.* **44**, 864–877. (doi:10.4319/lo.1999.44.3)
35. Deeks JJ, Higgins JP, Altman DG. 2011 Effect measures for dichotomous outcomes. In *Cochrane Handbook for Systematic Reviews of Interventions* (eds J Higgins, S Green),
36. Noordhuizen J, Frankena K, Thrusfield M, Graat E. 2001 *Application of quantitative methods in veterinary epidemiology*. Wageningen Pers.
37. R Core Team. 2016 R: a language and environment for statistical computing.
38. Robertson JL, Preisler HK, Russell RM. 2007 PoloPlus Probit and Logit analysis. *LeOra Softw.*
39. Sokal RR, Rohlf FJ. 1995 *Biometry: the principles and practice of statistics in biological research*. WH Freman and company: New York.
40. Wahl O, Ulm K. 1983 Influence of pollen feeding and physiological condition on pesticide sensitivity of the honey bee *Apis mellifera carnica*. *Oecologia* **59**, 106–128. (doi:10.1007/BF00388082)
41. Decourtye A, Lacassie E, Pham-Delegue M-H. 2003 Learning performances of honeybees (*Apis mellifera* L) are differentially affected by imidacloprid according to the season. *Pest Manag. Sci.* **59**, 269–278. (doi:10.1002/ps.631)
42. Meled M, Thrasyvoulou A, Belzunces LP. 1998 Seasonal variations in susceptibility of *Apis mellifera* to the synergistic action of prochloraz and deltamethrin. *Environ. Toxicol. Chem.* **17**, 2517–2520. (doi:10.1002/etc.5620171220)
43. Smirle MJ, Winston ML. 1987 Intercolony variation in pesticide detoxification by the honey bee (Hymenoptera: Apidae). *J. Econ. Entomol.* **80**, 5–8. (doi:10.1093/jee/80.1.5)
44. Baines D, Wilton E, Pawluk A, De Gorter M, Chomistek N. 2017 Neonicotinoids act like endocrine disrupting chemicals in newly-emerged bees and winter bees. *Sci. Rep.* 7, 10979. (doi:10.1038/s41598-017-10489-6)